Regulation of Poly(ADP-ribose) Polymerase 1 by Sumoylation and Investigation of Histone Poly(ADP-ribosyl)ation

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Simon Messner

aus

Österreich

Promotionskomitee

Prof. Dr. Dr. Michael O. Hottiger
(Vorsitz und Leitung der Dissertation)
Dr. Vikram Panse
Prof. Dr. Primo Schär
Prof. Dr. Roland Wenger

Zürich, 2010

Summary

Poly(ADP-ribose) polymerase 1 (PARP1) is an ubiquitously expressed chromatin-associated enzyme. It converts NAD^+ into poly(ADP-ribose), which is then attached to PARP1 itself or to other proteins. Poly(ADP-ribosyl)ation of proteins by PARP1 leads to alterations of protein functions, mainly by the negative charge of the ADP-ribose polymers. Thus, proper regulation of the enzymatic activity of PARP1 is absolutely required for the cell. PARP1 is involved in many cellular processes, such as transcription and maintenance of the genomic stability.

The aim of the thesis was to investigate whether PARP1 is sumoylated and how SUMO-modification would possibly influence the function of PARP1 *in vitro* and *in vivo*. Furthermore, we aimed to explore the ADP-ribosylation of PARP1 and other proteins, such as histones.

PARP1 was found to be modified by SUMO1 and SUMO3 *in vitro*, as well as *in vivo*. The sumoylation site was located within the auto-modification domain of PARP1 at lysine 486. Interestingly, SUMO-modification of PARP1 did not affect its enzymatic activity, but instead inhibited the acetylation of adjacent lysines by p300. The sumoylation deficient PARP1 K486R mutant exhibited higher acetylation levels *in vivo*. Furthermore, genetic complementation of cells with a SUMO-deficient PARP1-mutant revealed that SUMO-modification of PARP1 restrained transcriptional co-activator function of certain hypoxia-inducible genes.

In addition, the acetylation sites of PARP1, such as lysine 498, 521 and 524, were identified as ADP-ribose acceptor sites for the auto-modification of PARP1. This finding led to the investigation of ADP-ribose acceptor sites on histones. We found core histones H2A, H2B, H3 and H4 to be ADP-ribosylated by PARP1, as well as by PARP10. By site directed mutagenesis and mass spectrometry, the site of modification by PARP1 was mapped to specific lysines within the N-terminal tails of the core histones.

Taken together, these results reveal that the co-activator function of PARP1 is regulated through sumoylation as well as acetylation and that lysine residues functions as ADP-ribose acceptor sites within the auto-modification domain of PARP1, but also in core histone tails.

Zusammenfassung

Poly(ADP-ribose) polymerase 1 ist ein ubiquitär exprimiertes chromatin-assoziiertes Enzym. Es konvertiert NAD^+ zu Poly(ADP-ribose), welche an PARP1 selbst, oder an anderen Proteinen gebunden wird. Die Modifikation von Proteinen mit ADP-ribose durch PARP1 führt zu Änderungen der Proteinfunktion, hauptsächlich verursacht durch die hohe negative Ladung der ADP-ribose-ketten. Für die Zelle ist es daher absolut notwendig eine fehlerlose Regulation der enzymatischen Aktivität von PARP1 zu gewährleisten. PARP1 ist involviert in vielen zellulären Prozessen, wie zum Beispiel der Transkription und der Aufrechterhaltung der genomischen Integrität.

Das Ziel dieser Arbeit war es die Rolle von Sumoylierung für die Funktion von PARP1 zu finden, als auch die ADP-ribosylierung von PARP1 zu erforschen.

Es konnte gezeigt werden, dass PARP1 sowohl *in vitro* als auch *in vivo* mit SUMO1 und mit SUMO3 modifiziert wurde. Die Sumoylierung fand an Lysin 486 statt, innerhalb der Automodifizierungsdomäne von PARP1. Die SUMO Modifikation hatte interessanterweise keinen Einfluss auf die Automodifikation von PARP1 durch ADP-ribose, aber es inhibierte die Azetylierung von benachbarten Lysinen von PARP1 durch p300. Ausserdem wies eine sumoylierungsdefiziente PARP1 Mutante einen höheren Azetylierungsstatus *in vivo* auf. Eine genetische Komplementation von Zellen mit Sumoylierungsdefizientem PARP1 ergab, dass die SUMO-Modifikation von PARP1 die transkriptionelle Ko-aktivatorfunktion bei spezifischen Hypoxie induzierten Genen behinderte.

Weiters wurde gefunden, dass die Lysine 498, 521 und 524 von PARP1 ADP-ribosyliert werden. Dies führte zur Untersuchung von ADP-ribose Akzeptor Aminosäuren in Histonen. Die Histone H2A, H2B, H3 und H4 wurden von PARP1 und PARP10 ADP-ribosyliert. Durch den Einsatz von Punktmutationen und von Massenspektrometrie konnten Lysine am N-terminalen Histonende als ADP-ribose Akzeptor Aminosäuren identifiziert werden.

Zusammenfassend zeigen diese Resultate, dass die Ko-aktivatorfunktion von PARP1 durch Sumoylierung and Azetylierung reguliert wird und dass Lysine, innerhalb von PARP1, als auch im N-terminus von Histonen, ADP-ribose Akzeptorstellen sind.

Abbreviations

3-AB	3-amino-benzamide
ADP	adenosine diphosphate
ARH	ADP-ribosyl hydrolase
ARNT	aryl hydrocarbon receptor nuclear translocator
ASF1	anti-silencing function 1
ATP	adenosine triphosphate
BER	base excision repair
bHLH	basic helix-loop-helix
BRCT	BRCA1 C-terminus
CAF-1	chromatin assembly factor 1
CAIX	carbonic anhydrase 9
CBP	CREB binding protein
DNA	deoxynucleic acid
E2-25K	ubiqitin-conjugating enzyme E2K
EPO	erythropoietin
ERK2	extracellular signal-regulated kinase 2
FIH	factor inhibiting HIF
HAT	histone acetyl transferase
HDAC	histone deacteylase
HIF-1	hypoxia inducible factor 1
HIRA	histone regulator 1
HMG	high mobility group
HMG	high mobility group protein
HRE	hypoxia response element
HSF	heat shock factor
Hsp	heat shock protein
IKK	IκB kinase
IκB	inhibitor κB
LOXL2	lysyl oxidase like protein 2
LPS	lipopolysaccharide
MEF2	myocyte enhancing factor 2
NAD	nicotinamide adenine dinucleotide
Nap1	nucleosome assembly protein 1
NF-κB	nuclear factor κB
ODDD	oxygen dependent degradation domain
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly(ADP-ribose) polymerase
PAS	PER-ARNT-SIM
PDK1	pyruvate dehydrogenase kinase 1
PDSM	phosphorylation dependent sumoylation motif
PHD	prolyl hydroxylase
Pol II	RNA polymerase II
PTM	post-translational modification
RHD	Rel homology domain
ROS	reactive oxygen species
SIRT	sirtuin (silent mating type information regulation 2 homolog)
SUMO	small ubiquitin like modifier
TBP	TATA-binding protein
VHL	von Hippel-Lindau disease or protein
WGR	tryptophan-glycine-arginine motif
ZF	zinc finger

Contents

1 Introduction

1.1 Chromatin and transcription

The human genome has a size of $3.2x10^9$ nucleotide bases, which would equal approximately 2 m in length, if it were not tightly packed. Since each eukaryotic cell contains a full copy of the genome, condensation of the DNA is absolutely required. The compaction of the DNA is controlled by structural arrangements of the DNA with associated proteins, the histones and non-histone proteins, which form together the chromatin (1).

1.1.1 Structural Organization of Eukaryotic Chromosomes

The most abundant proteins in chromatin are the core histones H2A, H2B, H3, H4 and the linker histone H1 (1). They contain many positively charged amino acids, which allows them to interact with the negatively charged DNA backbone. The basic structural unit of chromatin is the nucleosome (2), which is made of a histone octamer that has 145-147 base pairs of DNA wrapped around it. This nucleoprotein complex is found every 200 ± 40 base pairs throughout the genome. The linker region between two nucleosomes can be bound by histone H1 and is variable in length (3). Each nucleosome core particle comprises a tetramer of H3 and H4 histones and two H2A-H2B dimers. Histones, which are not incorporated in the nucleosome, are often associated with chaperones. For example, unincorporated H3 is mainly found as dimeric unit with H4, associated with the histone chaperones CAF-1 (chromatin assembly factor 1) or HIRA (histone regulator A) within pre-deposition complexes (4). The H3-H4 heterodimeric complex associates also with the histone chaperone ASF1 (anti-silencing function 1) and promotes the nucleosome formation in synergy with CAF-1. When chromatin is extracted from nuclei at low salt concentrations, isolated chromatin resembles a "beads on a string" conformation. In this extended form, the string is composed of free linker DNA, which connects the nucleosomes. At physiological salt conditions, nucleosomes fold further into a chromatin fibre of 30 nm diameter (1). During cell division this structure can be additionally compacted by interaction with scaffolding proteins, to form the characteristic metaphase chromosome (5).

Although chromatin is highly heterogenous, it can be devided into the relatively uncondensed euchromatin, which includes most transcriptionally active regions, and the densely packaged heterochromatin. In heterochromatin, histone H1 stabilizes the higher order structure and compacts linear DNA overall by a factor of 30-40 (3). Chromatin structure imposes significant obstacles to all DNA-related metabolic processes including DNA-repair, recombination, replication, transcription and so forth. The dynamics of chromatin structure is tightly regulated through multiple mechanisms including chromatin remodeling (see chapter 1.1.2), histone modification (chapter 1.1.3) and histone variant incorporation (1.1.4).

1.1.2 Chromatin remodeling

Protein complexes that utilize ATP hydrolysis to alter histone-DNA contacts are generally referred to as chromatin-remodeling complexes (6). Upon binding of remodeling complexes to the nucleosome, they either slide the nucleosome to a different position, or transiently unwrap the DNA from the histone-octamer that enables binding of other protein complexes to DNA or facilitates histone exchange. In particular, histone H2A/H2B dimers are rather easily removed relative to H3 and H4 (2, 7). Histone eviction was observed upon co-operative binding of transcription factors (8), chromatin-remodeling complexes such as Swi/Snf (9) or actively transcribing RNA Polymerase II (Pol II) (10). Since displaced histones can bind to free DNA again, histone chaperones such as Asf1, Nap1 and nucleophosmin prevent re-binding of the histones (11-14).

1.1.3 Posttranslational modifications of histones

Core histones are mainly globular, except for their unstructured N-terminal tails. At least eight distinct types of posttranslational modifications (PTMs) have been described for these tails. The small chemical modifications, which include lysine acetylation, methylation of lysines and arginines and phosphorylation of serines and threonines, are characterized best. More complex modifications include proline isomerization, deimination of arginines, ubiquitination and sumoylation of lysines and ADP-ribosylation (see also chapter 1.3) (15). These modifications influence chromatin structure by regulating the binding of histone tails to other less abundant chromatin-associated proteins or to the DNA.

Given that modifications of histones by PTMs influence local chromatin structure as well as the recruitment of chromatin-modifying effectors, a histone code was proposed to

explain the correlations between histone PTMs and biological outputs (16-18). The hypothesis envisions that combinatorial histone PTMs encode the recruitment of specific chromatin-interacting components, which are determinant to a particular biological output, such as gene expression. Chromatin-interacting proteins are known to contain motifs which recognize and bind specific histone modifications. For example, bromodomains bind acetylated proteins and chromodomains interact with methylated proteins (19).

1.1.4 Histone variants

The general structure of chromatin has been conserved throughout evolution, which is also displayed in the amino acid sequence of histone proteins that are very similar between species. Nevertheless, in vertebrates minor histone variants have evolved, which can be incorporated into the nucleosome to exert specific functions (20). Histone variants differ from their more common counterparts only in a few amino acids. Thus, most sites of histone modifications are conserved (21) and may not affect nucleosome recognition by various chromatin-regulatory proteins. Histone variants can be divided into two major classes: replicative variants, which are synthesized in S-phase in a replication-coupled manner, whereas replacement forms are expressed either constitutively throughout the cell cycle or outside of the S-phase. Histone H2B and H4 are largely invariant, but Histone H2A and H3 are more disperse.

Histone H2A variants can be grouped into the replicative histone variants H2A.1, H2A.2 and the replacement histone variants H2Av (H2A.X/H2A.Z), Htz1 (H2A.Z), macroH2A and H2ABbd (4). Among these, H2A.X is the best studied histone variant. Upon treatment with DNA-double-strand break inducing agents, this variant becomes phosphorylated at its C-terminal SQ(E/D) motif (22, 23) and is subsequently named γH2A.X. The phosphorylation of H2A.X is crucial for the recruitment of DNA-repair proteins (24). Another H2A variant, macroH2A, contains a non-histone macro-domain at the C-terminus and is much larger than the canonical H2A (16). MacroH2A-containing nucleosomes are reported to be refractory to chaperone-mediated histone exchange, ATP-dependent remodeling, transcription factor binding or transcription initiation by Pol II (16, 25). Another histone variant, H2A.Z, is enriched at promoters that are poised for transcriptional activation (26). H2A.Z containing nucleosomes are resistant to chromatin remodeling and transcription elongation-related modifications (27). Therefore, rapid eviction of H2A.Z is needed for full transcriptional activation and elongation (28).

Histone H3 variants are grouped into the replicative H3.1 and H3.2 variants and the replacement H3.3 and CENP-A variants. CENP-A is the major H3 variant and is specific for centromeric chromatin and is essential for centromere function. Other H3 variants, such as H3.3, are involved in the regulation of transcription. For example, incorporation and removal of histone H3.3 is triggered by transcriptional activation (29) and is enriched in active chromatin (30).

Since higher order chromatin structure is typically repressive for transcription, the above described mechanisms (see 1.1.2 – 1.1.4) ensure chromatin dynamics, which is required to allow the transcriptional machinery to access the DNA. Transcription is a highly regulated process, which requires the concerted action of transcription factors (see 1.1.5) and their co-factors (see 1.1.6), which, in turn, are regulated by posttranslational modifications (see 1.3).

1.1.5 Transcription factors

Transcription factors are regulatory proteins with two basic functions, binding to DNA and activation or repression of transcription (31). These functions are often carried out by specific structural domains, which are in many cases conserved. One of these domains, the DNA-binding domain, is frequently used to classify transcription factors. For example, the transcription factor NF-κB belongs to the Rel family, which contain the conserved Rel homology domain (32). Another example is the transcription factor HIF-1, which binds DNA through a basic helix-loop-helix (bHLH) motif (33), found in a great number of DNA-binding proteins.

In addition to their modular domain structure, transcription factors often form homo- and heterodimers (34). On the one hand this facilitates the assembly of a complex, which is able to encircle DNA, on the other hand it creates vast combinatorial possibilities to regulate the affinity of proteins to specific DNA-sequences (34). Additional regulatory steps were described for nuclear import and export, posttranslational modifications, access to the binding site and through the use of numerous co-factors that modulate the activity of transcription factors.

1.1.6 Transcriptional co-activation and co-repression

Depending on their function, co-factors are either referred to as co-activators or co-repressors (31). Co-activators are proteins that dock on transcription factors and increase

the accessibility of chromatin-associated DNA for general transcription factors (35). Typical co-activators are chromatin-remodeling complexes, mediators or enzymes, which mediate histone modifications. Transcriptional co-factors influence transcription without necessarily binding DNA to exert their effect.

The best studied transcriptional co-activators are p300 and CREB binding protein (CBP) (36). CBP and p300 function as global transcriptional co-activators for at least 40 transcription factors (36). They co-activate transcription by different mechanisms. Firstly, they have been described as "molecular bridges" that mediate the interaction between sequence-specific transcription factors, Pol II and general transcription factors (37). Secondly, p300 and CBP serve as protein scaffold for the assembly of other chromatin modifying and remodeling complexes that increase the local concentration of co-factors around the transcription start sites (36). Thirdly, p300 and CBP acetylate themselves, histones and chromatin associated proteins, resulting usually in transcriptional activation (15).

Co-repressors, like co-activators, are multi-protein complexes. They achieve repression of transcription by different ways, including competitive binding with a co-activator to a transcription factor or inducing compaction of the chromatin-structure by histone deacetylation (31). A typical co-repressor is the CoREST1/HDAC2/LSD1 complex. This multiprotein complex directly deacetylates and demethylates histones, which consequently represses gene expression (38).

1.2 Hypoxia

Oxygen (O_2) is essential for the survival of all aerobic organisms. Proper oxygen levels are critical for the cell (39). Oxygen plays a crucial role as electron acceptor in the mitochondrial respiratory chain, enabling the generation of adenine trisphosphate (ATP) by oxidative phosphorylation (39). Disturbance of oxygen homeostasis can be caused by several pathologies, such as pulmonary and circulatory diseases. The level of oxygen supply depends largely on the distance from the nearest functional blood vessel. Usually, cells experience an oxygen level between 2.5% – 9% oxygen within the cell (40, 41). Hypoxia is defined as the state of reduced O_2 level below normal values (0.5% - 2.5% oxygen). Cells survive low levels of oxygen for several hours and only die if they are completely deprived of oxygen (anoxia). Hypoxia occurs at various physiological conditions, for example in the embryonic development, during adaptation to high altitudes and wound healing (40), but also in pathological conditions like ischemic diseases and cancer (42).

1.2.1 The hypoxia inducible factor (HIF) family of transcription factors

Although hypoxia generally inhibits transcription, a subset of genes is dramatically induced (33). The main transcription factor orchestrating the cellular response to low oxygen tension is the hypoxia inducible transcription factor (HIF) (33). HIF-1 is a heterodimer that consists of the HIF-1α subunit and a constitutively expressed HIF-1β subunit, which is also known as aryl hydrocarbon receptor nuclear translocator (ARNT) (39). Both subunits belong to the family of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain-containing transcription factors (43). The bHLH domain mediates the DNA-binding and the PAS-domain is responsible for the dimerization of HIF. In addition, HIF-1α contains an O_2-dependent degradation domain (ODDD) and two transactivation domains, including the N-terminal transactivation domains (NAD) and C-terminal transactivation domain (CAD).

1.2.2 Regulation of the transcriptional activity of HIF

The gene of HIF-1α is constitutively expressed and can be additionally increased by the transcription factor NF-κB, which is induced by LPS and cytokines (41), providing a link between hypoxic and innate immune responses.

The transcriptional activity of HIF-1α is mainly regulated at the posttranslational level. In presence of O_2, the overall levels of α-subunits are low, as a consequence of their rapid degradation by the proteasome. This degradation is a complex process and is initiated by the activity of the O_2-sensor prolyl hydroxylase domain protein (PHD). Proline hydroxylation by PHDs depends on the presence of the PHD co-factors oxygen, the co-substrate 2-oxoglutarate from the citric acid cycle, plus the co-factors Fe(II) and ascorbate (44, 45). In the presence of oxygen, PHDs hydroxylate HIF-1α at proline 402 and 564. The hydroxylated prolines are recognized by the von Hippel-Lindau (VHL) protein. This E3-ubiquitin ligase complex consists of elongin B, elongin C and cul2 and transfers ubiquitin to HIF-1α, which leads subsequently to proteasomal degradation of HIF-1α.

Under hypoxic conditions, PHD`s are inactive due to low oxygen levels and cannot hydroxylate HIF-1α, which leads to the stabilization of HIF-1α. Once HIF-1 protein is stable, it translocates to the nucleus, where it forms a heterodimer with HIF-1β and subsequently binds to the hypoxia response element (HRE) of its target genes. HIF-1α contains a N-terminal activation domain (NAD) and a C-terminal activation domain (CAD),

which act synergistically. Besides the above described O_2-dependent degradation mechanism, regulation of the HIF-1α CAD association with CBP/p300 is the second molecular switch controlling transcriptional activity of HIF-1 (46). The accessibility of the CAD for CBP/p300 is regulated through asparagine hydroxylation of HIF-1α by FIH-1 (factor inhibiting HIF-1) (47). Additionally, competition among transcription factors for CBP/p300 co-activator binding can also play a role, as shown for the transcription factor p53, which sequesters CPB/p300 from HIF-1 (48), leading to decreased HIF-1α target gene expression. In addition, posttranslational modifications of HIF-1α, such as acetylation (49) or sumoylation (50) modify the transcriptional activity of HIF-1α at the promoter (see also 1.3).

In summary, HIF-1α dependent gene expression is a rather complex mechanism that involves I) the regulation of HIF-1α mRNA levels; II) the regulation of HIF-1α protein stability; III) the regulation of transcriptional activation of HIF-1 at the promoter through FIH-1; and IV) the co-activation of HIF-1α dependent gene expression through different co-activators, such as CPB/p300.

1.2.3 Three examples for HIF-1 mediated responses to hypoxia

Mid- and long-term adaptations to hypoxic conditions are mediated through the expression of genes that are induced by HIF-1α. For example, HIF-1α induces the expression of pyruvate dehydrogenase kinase (PDK1) (42). PDK1 phosphorylates and inactivates the catalytic subunit of the pyruvate dehydrogenase (PDH), which results in the preferential conversion of pyruvate to lactate rather than to acetyl-CoA (51, 52). This leads to a reduction in oxidative phosphorylation and results in decreased ATP-production, a hallmark of hypoxia (39). The importance of PDK1 for the survival of hypoxia was demonstrated by overexpression of PDK1, which was sufficient to reduce ROS levels and prevented cell death (42).

Tumor cells experiencing prolonged hypoxia, decrease their pH both by production of lactic acid (due to the high glycolysis rates) and by CO_2 hydration catalyzed by the HIF-1α target gene carbonic anhydrase IX (CAIX) (53). Carbonic anhydrases catalyze the conversion of CO_2 to bicarbonate and protons and can thus be involved in the regulation of intracellular pH. They also secrete and export protons from cells and contribute additionally to acidosis, a hallmark of chronic hypoxia in solid tumors. The upregulation of CAIX is currently used as prognostic biomarker of tumor hypoxia (54). High expression of CAIX

correlates with poor survival. Increased levels of CAIX expression are seen in a high proportion of cervical carcinomas, kidney carcinomas and, to a lesser degree, in other types of human tumors, such as carcinomas of the breast, head, neck and lung, as well as tumors of the brain (54).

Another poor prognosis marker for the survival is the HIF-1 mediated increased expression of lysyl oxidase proteins (LOX) (55). These proteins were originally discovered to play a key role in the biogenesis of the connective tissue by catalyzing the crosslinking-formation in collagen and elastin (56). LOXL2 belongs to the four members of the lysyl-oxidase like proteins and was initially found to promote the invasiveness of tumors *in vivo* and *in vitro* (57). LOXL2 expression was upregulated in breast, colon, oesophageal, pancreatic, prostatic, head and neck squamous cell carcinoma (HNSCC) cell lines (58). More recently, it has been shown that LOXL2 interacts with the transcription factor snail, which promotes epithelial-mesenchymal transition and thus promotes, at least partly, the malignant progression of cancer cells (59).

1.3 Posttranslational modifications

Posttranslational modifications (PTMs) occur after the translation of a protein to regulate the protein functions (1). PTMs can be divided into several classes. Firstly, proteins can be altered by the addition of small functional groups, such as acetylation, phosphorylation (60), various lipids (61) and carbohydrates (62). Secondly, proteins can be modified by the addition of other polypeptides, examples include ubiquitination, neddylation, ISGylation and SUMOylation (63-65). Thirdly, the amino acids of proteins can be conversed to other amino acids by removal of terminal groups. An example for this modification is the conversion of arginine to citrulline by deimination (66). Finally, PTMs can involve large structural changes, like racemization of proteins by prolyl isomerization. Another example for the induction of a structural change is the proteolytic cleavage of proteins, such as the maturation of insulin by cleavage of its own precursor by site-specific endopeptidases, allowing the generation of active insulin (67).

1.3.1 Sumoylation

SUMO (**s**mall **u**biquitin like **mo**difier) polypeptides are approximately 10 kDa in size and resemble the three-dimensional structure of ubiquitin (68). They share only 20% sequence identity to ubiquitin and differ in their overall surface-charge distribution. In contrast to

ubiquitin, all SUMO polypeptides contain an unstructured stretch of 10-25 amino acids at their N-termini. The human genome encodes four SUMO proteins (SUMO1-4). SUMO1-3 are ubiquitously expressed, whereas SUMO4 is expressed in a tissue specific manner (69) and is less well-studied (64). While the mature form of SUMO2 and SUMO3 are 97% identical, they share only 50% sequence homology with SUMO1 (64). Furthermore, SUMO1 is mainly attached as monomeric unit to substrate proteins *in vivo*, whereas SUMO2 and SUMO3 are able to form poly-SUMO chains. Based on the low sequence homology and the different expression patterns of SUMOs, it is not surprising that SUMO1 and SUMO2/3 are conjugated to different target proteins *in vivo* and exert distinct functions (70-72)

The reversible cycle of SUMO conjugation and de-conjugation of target proteins is described in Fig. 2. It is similar to the ubiquitin conjugation and involves the activation of the SUMO-precursor *(1)*, conjugation to a SUMO E1-activating enzyme *(2)*, transfer to the SUMO E2-conjugating enzyme *(3)* and finally the transfer of SUMO to a lysine residue of a target protein, often with the help of a SUMO E3-ligase *(4)*. Finally, the SUMO moiety can also be cleaved off from the target protein by SUMO proteases *(5)* (73).

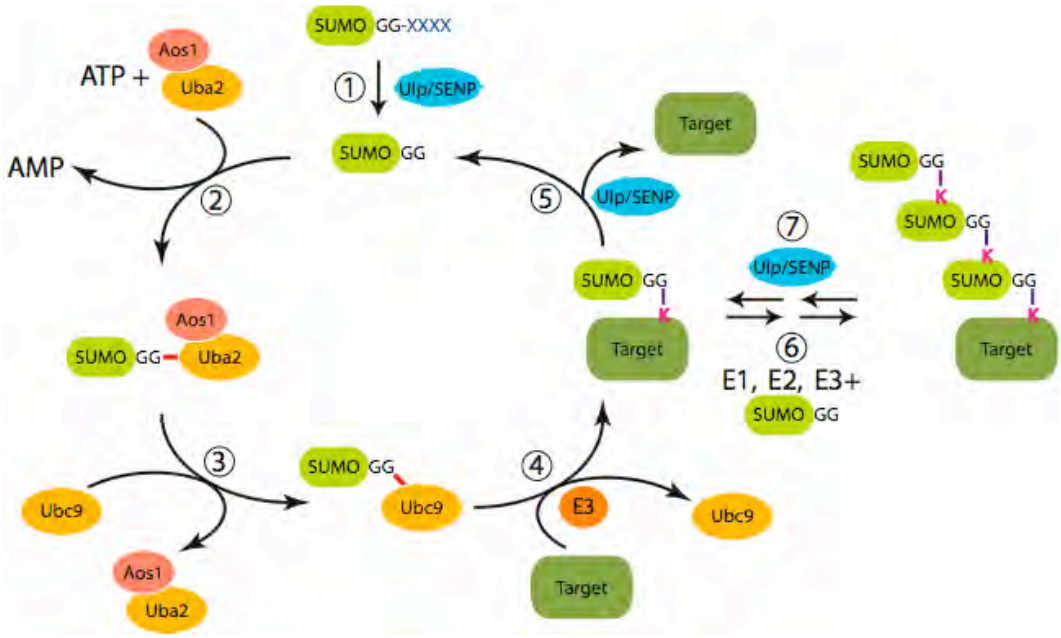


Figure 2: SUMO proteins undergo post-translational maturation, catalyzed by Ulp/SENPs, to reveal a C-terminal di-glycine motif (Step 1). Mature SUMOs undergo ATP-dependent activation, resulting in a thiolester linkage between the C-terminal di-glycine and their activating enzyme, Uba2/Aos1 (Step 2). The thiolester is transferred to their conjugating enzyme, Ubc9 (Step 3). Ubc9 acts in concert with SUMO ligases/E3 enzymes to form an isopeptide linkage between the SUMO C-terminus and an ε-amino group of a lysine within the target protein (Step 4). SUMOs can be removed from conjugated species by the action of Ulp/SENPs (Step 5). In some cases, SUMO chains can be formed through linkage of additional SUMO moieties to previously conjugated SUMOs (Step 6). While it is possible that multiple Ulp/SENPs may disassemble SUMO chains (Step 7), members of the Ulp2 family appear to be specialized for this reaction. Adapted from (73).

Many proteins are sumoylated at a SUMO-consensus motif, which has been shown to resemble the amino acid sequence ΨKxE (in which Ψ is an aliphatic branched amino acid and x is any amino acid) (74). The SUMO E2 enzyme Ubc9 recognizes this sequence and transfers the SUMO-moiety onto a lysine residue of a substrate protein. Extensions of the SUMO-consensus motif have been described. For example, for the phosphorylation-dependent sumoylation motif (PDSM), the classical SUMO consensus motif is followed by a phosphorylated serine and a proline residue. This extended motif is found in heat shock factor-1 (HSF1), MEF2 and several other proteins (75). However, some SUMO acceptor sites have been reported not to contain any of the described motifs, such as K14 in human E2-25K (76). In addition, a non-covalent SUMO interacting motif (SIM) was identified, which contains a hydrophobic core, flanked by acidic and/or serine residues (77). The functional consequences of sumoylation are diverse and depend on the modified protein. In most cases, sumoylation creates new interaction surfaces or covers existing ones (64).

An increasing number of papers report regulation of sumoylation by cellular stresses and suggest an important role for this modification in cellular response to environmental cues. Along this line, heat shock increases global sumoylation levels dramatically (70), as shown for the elevated SUMO modification of more than 700 proteins (78). Another cellular stress that induces global sumoylation is hypoxia. Elevated transcription of the SUMO1 gene starts within 4-8 hours after hypoxia and correlates with increased levels of protein sumoylation after hypoxia (79, 80). For example, the transcription factor HIF-1α is sumoylated by SUMO1 upon hypoxic induction. The consequences of this increase in HIF-1α sumoylation are still a matter of debate (50). In some reports, sumoylation of HIF-1α leads to its stabilization and increased transcriptional activation of target genes (81, 82). In another study, however, sumoylated HIF-1α is degraded by the VHL ubiquitin ligase complex and only a low level of active HIF-1α is maintained during hypoxia through the SUMO-deconjugation of HIF-1α by the SUMO-protease SENP1 (83). A different mechanism of HIF-1 regulation by sumoylation was described recently (84). In this study, modest reactive oxygen species (ROS) levels induced SENP3 (Sentrin specific protease 3) translocation from the nucleolus to the nucleus (84). Once in the nucleus, SENP3 desumoylates p300, which enables complex formation between p300 and HIF-1α, thus leading to transcriptional activation of HIF-1 target genes.

1.3.2 Acetylation

Lysine acetylation is an ancient reversible PTM that is conserved from prokaryotes to humans (85). The transfer of an acetyl-group from acetyl-coenzyme A (CoA) onto the ε-

amino-group of the target lysine neutralizes the positive charge of the lysine (Fig. 1). The acetylation impairs the ability of lysines to form hydrogen bonds with other amino acids, thereby affecting protein-protein, protein-DNA and protein-RNA interactions (86).

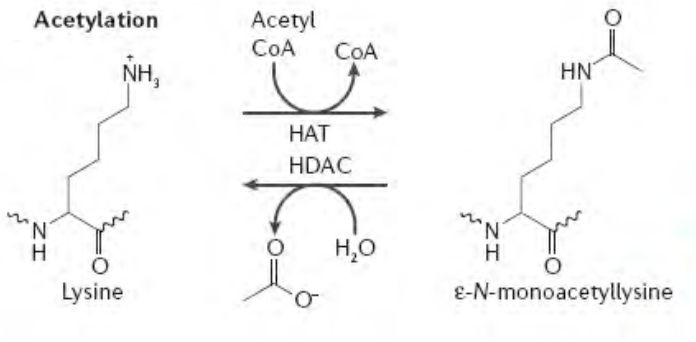


Figure 1. ε-N-lysine acetylation. Transfer of an acetyl group from acetyl-coenzyme A (CoA) onto the ε-N-group of a lysine residue in protein x by histone acetyltransferases (HAT). The reverse reaction is catalyzed by histone deacetylases (HDAC) and removes the acetyl-group. Adapted from (60).

Acetylation is known to regulate different nuclear processes, such as transcription through acetylation of histone proteins, which loosens the chromatin structure and thus often correlates with activated gene expression. Upon initiation of transcription, activators recruit histone acetyltransferases (HATs) to acetylate gene-specifically the chromatin and enhance transcription, while repressors, such as histone deacetylases (HDACs), deacetylate histones and inhibit transcription. Histone acetylation also regulates other nuclear processes, such as DNA replication, recombination and repair (15).

Histone acetyltransferases catalyse the acetylation of lysine residues. Dozens of proteins with HAT activity have been described so far. HATs can be divided into two groups: the cytoplasmic B-type HATs and nuclear A-type HATs. In general, nuclear HATs are associated with transcriptional activation and are present in euchromatin. Lysine acetyltransferases are classified into different superfamilies, such as the superfamily of Gcn5/PCAF, p300/CBP and the MYST protein containing complexes. While Gcn5/PCAF and p300/CBP histone acetyltransferases mainly function as transcriptional co-activators, emerging evidence suggests that MYST proteins, such as Esa1, Sas2, MOF, TIP60, MOZ and MORF, play diverse roles in various nuclear processes (87). As for example, mammalian Tip60 plays an important role in apoptosis and DNA repair (88, 89). Most of the histone acetyltransferases were first shown to acetylate histones (Table 1), but later HATs have been shown to acetylate non-histone proteins as well.

Acetyltransferase	**Residues modified**
HAT1	H4 (K5, K12)
CPB/p300	H3 (K14, K18), H4 (K5, K8), H2A (K5), H2B (K12, K15)
PCAF/GCN5	H3 (K9, K14, K18)
Tip60	H4 (K5, K8, K12, K16)
HB01 (ScESA1, SpMST1)	H4 (K5, K8, K12)
ScSAS3	H3 (K14, K23)
ScRTT109	H3 (K56)
ScSAS2 (SpMST2)	H4 (K16)
Deacetylase	
SIRT2	H4 (K16)

Table 1: HATs and HDACs that modify histones. Only enzymes with specificity for one or a few sites have been included in the table, along with the sites they modify. Human and yeast enzymes are shown. The yeast enzymes are distinguished by a prefix: Sc (Saccharomyces cerevisiae) or Sp (Saccharomyces pombe). Adapted from (15).

Histone deacetylases remove the acetyl group from acetylated proteins (87). The activity of HDACs is tightly controlled through protein-protein interactions and posttranslational modifications (90). With the exception of one HDAC (HDAC8), functional HDACs are never found as single monomeric polypeptides, but are rather found in high molecular weight multi-protein complexes. They often associate with specific co-regulators as well as with other chromatin modifying enzymes (90). Mammalian histone deacetylases are divided into four classes.

Class I HDACs consist of the members HDAC1-3 and HDAC8. They are part of the ubiquitously expressed mSin3A, NURD/Mi2/NRD and CoREST co-repressor complexes (91).

Class II HDACs are further divided according to their sequence homology and domain organization into the subclass IIa (HDAC4, 5, 7, 9) and IIb (HDAC6, 10). Class IIa HDACs are characterized by tissue specific expression and stimulus-dependent nucleo-cytoplasmic shuttling. Class IIb HDACs are distinct to class IIa due to a duplication of the catalytic domain (90).

Class III HDACs are named Sir2-like proteins or SIRTs. These proteins represent NAD^+-dependent deacetylases (SIRT1-7). They participate in a wide range of cellular processes including DNA-repair and determination of life span (92). SIRT1 deacetylates transcription factors, such as PGC-1α, FOXO1 and TORC2 (93), which are important in cell metabolism and implicated in aging. SIRT2 activity is necessary for proper cytokinesis and inhibition of SIRT2 was protective in a *Drosophila* model of Parkinson's disease (93). SIRT6

deacetylates H3 at lysine 9, which is implicated in maintenance of the genomic integrity (94). Additionally, SIRT6 was shown to possess auto-ADP-ribosylation activity (95).

Finally, class IV is represented by HDAC11, which is phylogenetically different from both class I and class II HDACs and is therefore regarded as separate class (90).

1.3.3 Crosstalk of sumoylation with acetylation

Since sumoylation and acetylation both target lysine residues, they might compete for the same residue. Modification of the same residue was observed for transcription factor Sp3 (96) and for the transcriptional co-activator p300. Deacetylation of p300 by SIRT1 is required for efficient sumoylation at the same lysine residue (97). It is currently not known, whether lysine acetylation frequently overlaps with SUMO acceptor sites (98). Additionally, an interplay between acetylation, sumoylation and phosphorylation was observed for the transcription factor MEF2A. Dephosphorylation of serine 408 in MEF2A, which is located in the phosphorylation dependent SUMO-motif (PDSM), results in a switch from sumoylation to acetylation at lysine 403 of MEF2A (99). Consequently, sumoylation of MEF2A is inhibited not only by the lack of a phosphate on serine 408, but also by direct competition of acetylation and sumoylation for lysine 403. Surprisingly, it has been observed that HDAC4 enhances sumoylation of a variety of substrates (100-102), such as MEF2 (103). However, deacetylation of the lysine residue within the SUMO-consensus site of MEF2 is not catalyzed by HDAC4, but rather by SIRT1 (103).

1.4 Poly(ADP-ribose) polymerase 1 (PARP1)

1.4.1 Structure of PARP1

PARP1 is a chromatin associated, ubiquitously expressed enzyme (104). It converts NAD^+ into poly(ADP-ribose), which is then attached to PARP1 itself or to other proteins (105). PARP1 contains two zinc fingers at the N-terminus that were reported to bind to various DNA strand breaks (Fig. 3). Recently a third zinc finger within the DNA binding domain was discovered (106). The BRCT domain is named after the Breast Cancer Suppressor Protein-1 (BRCA1) carboxy-terminal domain and is found within many DNA damage, DNA repair and cell cycle checkpoint proteins (107). The WGR domain belongs to the catalytic domain and is named after the most conserved central motif (W/G/R) of the domain. The WGR is found in a variety of polyA polymerases and other proteins of unknown function (108). The

Diphtheria toxin-related catalytic domain of PARP1 is responsible for the formation of poly(ADP-ribose). By site directed mutagenesis it was revealed, that glutamic acid residue 988 in the catalytic core of PARP1 is important for the formation of ADP-ribose polymers (109). Mutation of gluatamic acid 988 to a lysine severly impaired PARP1´s enzymatic activity. The mutant displayed only very weak mono(ADP-ribosyl)ation activity and completely lacked poly(ADP-ribose) formation activity (109).

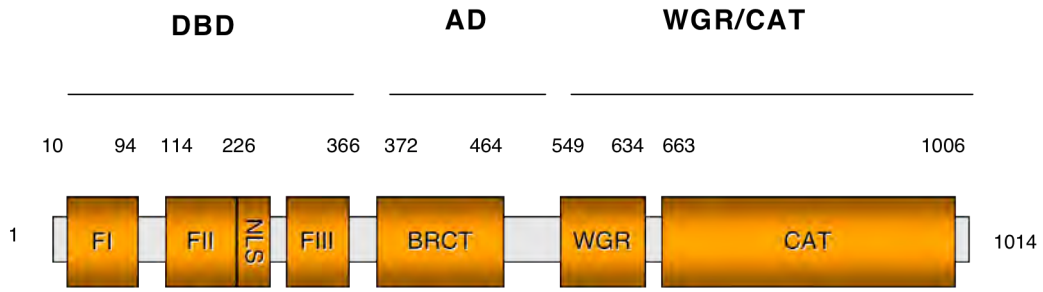


Figure 3: Domain structure of human PARP1. DBD: DNA-binding domain; AD: Auto-modification domain; FI-FIII: Zincfinger I-III; NLS: Nuclear localization signal; CAT: catalytic domain; BRCT: BRCA1 C-terminal domain; WGR: named after the central W/G/R motif.

PARP1 is the founding member of the PARP family, which consists of PARP1-6. They are characterized by the presence of an active site glutamate in the catalytic core domain. In addition to the 6 PARP members, 11 new PARP-like genes have been identified (108), all sharing the characteristic catalytic domain, but not the conserved glutamate E988 of PARP1. Although no poly(ADP-ribosyl)ation has been described for them, some are able to catalyze mono(ADP-ribosyl)ation (104).

1.4.2 The poly(ADP-ribosyl)ation cycle

Poly(ADP-ribosyl)ation is observed in multicellular eukaryotes only, and is characterized by the formation of long chains of ADP-ribose, linked by a glycosydic bond (104). The enzymatic activity of PARP1 is highly activated by DNA-strand breaks (110). Furthermore, PARP1 was reported to be activated in a DNA-independent manner by phosphorylated ERK2 (extracellular signal-regulated kinase 2) (111). PARP1 is able to synthesize poly(ADP-ribose) and mainly modifies itself, but also other proteins such as histones, high-mobility-group (HMG) proteins and several enzymes involved in DNA metabolism (104). Poly(ADP-ribosyl)ation alters the physical properties of target proteins, since the polymers are highly negatively charged and very bulky.

At least three distinct enzymatic activities were postulated to be required for the synthesis of free or protein-associated linear and branched poly(ADP-ribose) (108). I) Initiation: covalent auto-mono(ADP-ribosyl)ation or mono(ADP-ribosyl)ation of a substrate protein, which serves as initiator site for poly(ADP-ribosyl)ation; II) Elongation: involves the catalysis of a 2'-1'' glycosydic bond, whereby the covalently bound mono(ADP-ribose) from the initiation serves as acceptor site; and III) Branching: occurs on average after 20 ADP-ribose units.

The major fraction of PARP1-generated poly(ADP-ribose) has a half-life as low as 1 min while the residual fraction has a half-life of 6-10 min (108). The release of the enzyme-bound branched poly(ADP-ribose) is either mediated through intrinsic poly(ADP-ribosyl)protein-hydrolase activity of PARP1, or through poly(ADP-ribose) glycohydrolase activities, most likely mediated by poly(ADP-ribose) glycohydrolase (PARG). PARG is located in the nucleus and possesses both endoglycosidase and exoglycosidase activities (108), which are responsible for the hydrolysis of glycosydic ribose-ribose bonds within the polymer and at the end of the ADP-ribose chain, respectively. Branched and short polymers are more slowly degraded by PARG than long and linear poly(ADP-ribose) polymers.

Mammalian cells contain several different PARG-like genes. For example, ARH1 (ADP-ribose-arginine protein hydrolase 1) has been described to hydrolyze the ADP-ribose-arginine bond, thus generating free mono(ADP-ribose) (112). However, the closely related enzyme ARH3 possesses no arginine-ADP-ribose hydrolyzing activity, but instead displays PARG like activity, which leads to the degradation of poly(ADP-ribose) (113, 114). Of note, ARH1 and ARH3 are mainly located in the cytoplasma and on the cell surface and are therefore postulated not to be responsible for degradation of PARP1-generated poly(ADP-ribose).

1.4.3 PARP1 knockout mice

Several PARP1 knockout ($PARP1^{-/-}$) mouse models have been generated (115, 116). $PARP1^{-/-}$ mice are viable and fertile and show some interesting stress-phenotypes (117). Among these, $PARP1^{-/-}$ mice are protected against cerebral ischemia (118). Cerebral ischemia is characterized by insufficient blood flow in the brain, which causes brain damage. In mouse models of ischemia-reperfusion, which is characterized by tissue damage through restricted blood flow and subsequent reperfusion of the tissue (119), $PARP1^{-/-}$ mice showed a protected phenotype (120). $PARP^{-/-}$ mice were also reported to be protected against streptozotocin induced diabetes (121, 122). Streptozotocin is a toxic

substance that induces pancreatic beta cell death and is used to mimic type-1 diabetes in animal models (123). Another protective phenotype was observed when PARP1$^{-/-}$ mice were treated with high doses of lipopolysaccharides (LPS). Most of the PARP1$^{-/-}$ mice survived, whereas their wild-type counterparts died of a septic shock (124, 125).

1.4.4 PARP1 and transcription

PARP1 is localized in the nucleus and specifically enriched in MCF-7 cells at the promoters of approximately 90% of Pol II transcribed genes (126). The accumulation of PARP1 to actively transcribed promoters may occur as a consequence of the transcription process, for example recruitment of PARP1 to histone modifications, recruitment to oxidized or cleaved promoter DNA or special DNA structures (127, 128). Based on this localization, PARP1 could exert stimulatory or inhibitory effects on transcription. Global transcription analysis by microarray techniques of PARP1$^{-/-}$ mice compared to wild-type mice revealed that approximately 3.5% of the transcriptome in embryonic cells is regulated by PARP1, with approximately 60-70% of the genes being positively regulated (129). These genes encode for proteins involved in metabolism, stress response, signal transduction, cell cycle control and transcription. Multiple molecular modes of transcriptional regulation by PARP1 have been proposed (reviewed in (130)). Firstly, PARP1 can modulate chromatin structure by binding to nucleosomes, modifying histones, or regulating the composition of chromatin (131-133). Secondly, PARP1 can act as an enhancer-binding factor where it may bind to specific sequences or structures in the DNA (134, 135). Thirdly, PARP1 can act as a classical co-activator or co-repressor by binding and stabilizing the general transcription factors, the pre-initiation complex and/or specific transcription factors, to induce gene expression (111, 136-139). Fourthly, PARP1 can act as a component of insulators, which act to limit the effects of enhancers on promoters or by preventing the spread of heterochromatin (140). One can imagine that all these modes act either independently, or work in concert with each other. In summary, all of these possibilities may contribute to ensure proper tissue- and signal-specific gene expression.

The resistance of PARP1$^{-/-}$ mice to LPS-induced septic shock is most probably due the impaired induction of NF-κB dependent genes in PARP$^{-/-}$ mice (136). This is consistent with the notion that PARP1 acts as classical co-activator of NF-κB induced gene expression (136). Co-activation of NF-κB by PARP1 depends on the acetylation of PARP1 by p300 at specific lysine residues (lysines 498, 505, 508, 521 and 524). Acetylation of PARP1 facilitates its interaction with NF-κB and enables the induction of NF-κB dependent gene

expression (137). In addition, HDAC1, 2 and 3 were shown to deacetylate PARP1, which abrogated PARP1 dependent NF-κB interaction (137, 141).

1.4.5 PARP1 and DNA-repair

Since the enzymatic activity of PARP1 has been shown to be induced 10-500 fold by ionizing radiation, alkylating agents or oxidative stress, it has been suggested that PARP1 is a sensor of DNA-damage (105). Indeed, PARP1 is able to recognize DNA lesions by its DNA-binding domain, which initiates auto-modification of PARP1 (105, 142). It has been proposed that PARP1 influences DNA damage signalling by the recruitment of DNA-repair proteins to the DNA lesion through auto-poly(ADP-ribosyl)ation (143). In addition, PARP1 was reported to relax chromatin through poly(ADP-ribosyl)ation of histones and thus to facilitate access to sites of DNA-damage (133, 144, 145). However, no direct evidence for the involvement of PARP1 in the execution of DNA-repair was demonstrated so far (146). Interestingly, PARP1-mediated poly(ADP-ribosyl)ation recruits the histone variant macroH2A1.1 to sites of DNA-damage, which transiently compacts chromatin and reduces recruitment of DNA damage factors Ku70-Ku80 (147).

1.4.6 PARP1 and cancer

A hallmark of cancer is the accumulation of mutations in the DNA, which can lead to aberrant expression of important proteins. Since PARP1 has been suggested to play a key role as DNA-damage sensor (see 1.4.5), it has been proposed that the activity of PARP1 would be beneficial for the prevention of cancer. Indeed, $PARP1^{-/-}$ mice appear to be sensitive to alkylating agents (148). For example, double knockout mice for PARP1 and p53, but not the corresponding single knockout mice develop spontaneously brain tumors (149). In contrast, a different strain of $PARP1^{-/-}/p53^{-/-}$ mice shows in general attenuated tumor formation (150). In agreement with a protective role of PARP1 for tumor formation, PARP inhibitor treatment of mice suppresses tumorigenesis in TPA-induced skin cancer model (151). These contradictions could be explained by differences between various cancer types and the fact that PARP1 has several cellular functions, which could differently contribute to cancer formation (110, 152). Several PARP inhibitors are currently evaluated in clinical trials (153-155). They are used, in particular, for the treatment of tumors with BRCA1 or BRCA2 deficiency, since PARP inhibitors were reported to induce synthetic lethality in these cell lines (156, 157). In addition to BRCA1/BRCA2 deficient tumor cells, also cell lines with mutated PTEN (phosphatase and tensin homolog) show comparable

sensitivity towards PARP inhibitors (158), further broadening the potential use of PARP inhibitors in the treatment of cancer.

2. Aim of the thesis

PARP1 is an ubiquitously expressed, chromatin-associated protein and involved in many cellular processes. Understanding the regulation of its cellular function is of particular interest. One possible way to regulate the function of PARP1 is through posttranslational modifications, such as sumoylation, acetylation or ADP-ribosylation.

The aim of the thesis was to investigate whether PARP1 is sumoylated and how SUMO-modification would possibly influence the function of PARP1 *in vitro* and *in vivo*. Furthermore, we aimed to explore the mechanism of ADP-ribosylation by PARP1 and other proteins, such as histones.

3. Results

3.1 Overview of published and submitted manuscripts

3.1.1 Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional co-activator function.

Authors: Simon Messner, David Schuermann, Matthias Altmeyer, Ingrid Kassner, Darja Schmidt, Primo Schär, Stefan Müller and Michael O. Hottiger

Journal: FASEB J (2009) 23(11): 3978-89

Link: http://www.fasebj.org/cgi/content/full/23/11/3978

Contribution: Planning and performance of experiments in Fig. 1A,C-E, Fig. 2A-E, Fig. 3A,B,D, Fig. 4A-D, Fig. 5A-D, Suppl.Fig. 1A-C, Suppl.Fig. 2A-C, Suppl.Fig. 3A, Suppl. Fig. 5A-E; M.O.H supervised the project and wrote together with S.M. the manuscript.

3.1.2 PARP1 ADP-ribosylates lysine residues of the core histone tails

Authors: Simon Messner, Matthias Altmeyer, Hongtao Zhao, Bernd Roschitzky, Peter Gehrig, Dorothea Rutishauser, Andrea Pozivil, Danzhi Huang, Amedeo Caflisch and Michael O. Hottiger

Journal: Nucleic acid research (2010), 1-13

Link: http://nar.oxfordjournals.org/cgi/content/abstract/gkq463

Contribution: Planning and performance of experiments in Fig. 1A,B,F; Fig. 3A-D, Fig. 4D-G; Fig. 5A-B, Suppl.Fig. 1A,B; Suppl.Fig. 2A,B,E,G; S.M., B.R., D.R. and P.G analyzed the results of mass spectrometry; S.M, M.A., A.C and M.O.H wrote the manuscript.

3.1.3 Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites (Title page)

Authors: Matthias Altmeyer, Simon Messner, Paul O. Hassa, Monika Fey and Michael O. Hottiger

Journal: Nucleic Acids Research (2009) 37(11): 3723-38

Link: http://nar.oxfordjournals.org/cgi/content/full/37/11/3723

Contribution: Planning and performance of experiments leading to the identification of lysines as ADP-ribose acceptor sites in Fig. 6C-E. Analysis of the results, proofreading of the manuscript.

3.1.4 PARP1 regulates tumor progression by co-activating HIF-1-dependent gene expression (Title page)

Authors: Michael Elser, Lubor Borsig, Paul O. Hassa, Suheda Erener, Simon Messner, Taras Valovka, Stephan Keller, Max Gassmann and Michael O. Hottiger

Journal: Molecular Cancer Research (2008) 6(2): 282-90

Link: http://mcr.aacrjournals.org/content/6/2/282.long

Contribution: Planning and performance of experiments in Figure 5C and 5D.

3.1.5 Histone ADP-ribosylation revisited

Authors: Simon Messner and Michael O. Hottiger

Journal: submitted for publication

Link: not yet available

Contribution: Literature research, writing and proofreading of the paper.

The FASEB Journal article fj.09-137695. Published online July 31, 2009.

Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function

Simon Messner,[*,†] David Schuermann,[‡] Matthias Altmeyer,[*,†] Ingrid Kassner,[*,†] Darja Schmidt,[§] Primo Schär,[‡] Stefan Müller,[§] and Michael O. Hottiger[*,1]

[*]Institute of Veterinary Biochemistry and Molecular Biology and [†]Life Science Zurich Graduate School, Molecular Life Science Program, University of Zurich, Zurich, Switzerland; [‡]Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Basel, Switzerland; and [§]Department of Molecular Cell Biology, Max Planck Institute of Biochemistry, Martinsried, Germany

ABSTRACT **Poly(ADP-ribose) polymerase 1 (PARP1) is a chromatin-associated nuclear protein and functions as a molecular stress sensor. At the cellular level, PARP1 has been implicated in a wide range of processes, such as maintenance of genome stability, cell death, and transcription. PARP1 functions as a transcriptional coactivator of nuclear factor κB (NF-κB) and hypoxia inducible factor 1 (HIF1). In proteomic studies, PARP1 was found to be modified by small ubiquitin-like modifiers (SUMOs). Here, we characterize PARP1 as a substrate for modification by SUMO1 and SUMO3, both *in vitro* and *in vivo*. PARP1 is sumoylated at the single lysine residue K486 within its automodification domain. Interestingly, modification of PARP1 with SUMO does not affect its ADP-ribosylation activity but completely abrogates p300-mediated acetylation of PARP1, revealing an intriguing crosstalk of sumoylation and acetylation on PARP1. Genetic complementation of PARP1-depleted cells with wild-type and sumoylation-deficient PARP1 revealed that SUMO modification of PARP1 restrains its transcriptional coactivator function and subsequently reduces gene expression of distinct PARP1-regulated target genes. Messner, S., Schuermann, D., Altmeyer, M., Kassner, I., Schmidt, D., Schär, P., Müller, S., and Hottiger, M. O. Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function. *FASEB J.* 23, 000–000 (2009). www.fasebj.org**

Key Words: NAD · SUMO · hypoxia · PARP-1

Poly(ADP-ribose) polymerase 1 (PARP1) is an abundant nuclear chromatin-associated multifunctional enzyme found in higher eukaryotes that belongs to a family of 5 "*bona fide*" PARP enzymes (1). PARP1 has an amino-terminal DNA-binding domain (DBD) containing 3 zinc finger motifs, as well as a central automodification domain (AMD), which functions as a target of direct covalent automodification. The carboxyl-terminal catalytic domain polymerizes linear or branched chains of ADP-ribose from the donor nicotinamide adenine dinucleotide (NAD^+). ADP-ribose is mainly attached on PARP1, but also other proteins are modified (2). Together, the DBD and the automodification domain allow PARP1 to interact with genomic DNA and chromatin. Although originally characterized as a key factor in DNA single strand-break repair, a wealth of studies over the past decade have demonstrated a role of PARP1 in the regulation of gene expression under basal, signal-activated, and stress-activated conditions (1, 3). Recent studies have highlighted the role of PARP1 in distinct modes of transcriptional regulation and provided novel insight into the cellular signaling systems that interface with PARP1 in the nucleus (4).

The basal enzymatic activity of PARP1 is very low, but it is stimulated dramatically under conditions of cellular stress (2, 3). Activation of PARP1 results in the synthesis of poly(ADP-ribose) (PAR) from NAD^+ and the release of nicotinamide as a reaction by-product (1). Following PARP1 activation, intracellular PAR levels can rise 10- to 500-fold (1), caused by a mechanism that remains to be resolved. Very recently, we identified 3 lysine residues in the automodification domain of PARP1 as acceptor sites for auto-ADP-ribosylation (5). PARP1 is the main acceptor for poly(ADP-ribosyl)ation *in vivo*, and automodification of PARP1 abolishes its affinity for NAD^+ and DNA (5). Remarkably, the same 3 ribosylated lysines (K498, K521, K524) were previously identified as targets for acetylation by the histone acetyltransferase p300 (6). Acetylation of PARP1 has been reported to be important for its transactivation activity (6). Recently, we also highlighted the role of PARP1 as a transcriptional coactivator of hypoxia inducible factor 1-α (HIF1-α). On hypoxic induction of cells, PARP1 was shown to interact with HIF1-α and to regulate the transcriptional activity of HIF1-α-dependent genes (7).

[1] Correspondence: Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail: hottiger@vetbio.uzh.ch

doi: 10.1096/fj.09-137695

0892-6638/09/0023-0001 © FASEB

Another post-translational protein modification in response to cellular stresses is the conjugation of small ubiquitin-like modifiers (SUMOs) (8). SUMOs regulate diverse cellular processes, including cell-cycle progression, genome stability, intracellular trafficking, and transcription (9, 10). In many cases, SUMO conjugation alters localization and/or activity of the substrate by providing a new protein-protein interaction interface. However, in certain cases, SUMO modification can also prevent distinct protein-protein interactions. Mammalian cells express three SUMO paralogs: SUMO2 and SUMO3, which are 96% identical and only differ by three N-terminal residues, and SUMO1, which is 45% identical to SUMO2/3. Moreover, SUMO2/3 proteins are able to form chains, which SUMO1 cannot (11). Although virtually all of the SUMO1 is engaged in conjugates, there is a free pool of the more abundant SUMO2/3 that is utilized when cells are stressed by heat shock or ethanol exposure (12). It is clear that proteins can be modified selectively by SUMO1 and SUMO2/3. Growing evidence suggests that SUMO2/3 and SUMO1 have some unique biological functions (12–14).

SUMO family proteins are conjugated to target lysines *via* a cascade of the E1-activating enzyme (SAE1/SAE2), the E2-conjugating enzyme Ubc9, and E3 SUMO ligases (8, 10). The SUMO E2 protein Ubc9 often recognizes the consensus sequence ΨKxE/D (where Ψ is a large hydrophobic amino acid, such as isoleucine or valine, and x is any amino acid) in the target protein and catalyzes SUMO conjugation (8). Generally, sumoylation with SAE1/SAE2 and Ubc9 only is rather inefficient, and additional proteins known as SUMO E3 ligases are often required to accelerate this reaction (10). A family of deconjugation enzymes, SENPs, is responsible for the removal of SUMO from target lysines (15), which accounts for the transient nature of this modification. In human cells, six members of this family (SENP1-3 and SENP5-7) have been identified. Importantly distinct members exhibit paralog specificity and show a characteristic subcellular localization, indicating that spatial control is an important regulatory concept of SENP activity.

Several proteomic studies to identify substrates for SUMO conjugation have been reported (16–18). In this context, PARP1 was detected to be sumoylated in HEK293 cells and in K562 cells. SUMO modification of proteins that regulate transcription has been associated with dynamic regulation of gene expression (9, 19). A large number of transcriptional regulators, including transcription factors, cofactors, and chromatin-modifying enzymes, have been found to be substrates of SUMO modification. Generally, a SUMO-modified factor exists in a dynamic distribution between the SUMO-modified and unmodified forms, and although the SUMO-modified form of a protein is often difficult to detect, it can have a great impact on transcriptional activation (9, 10). Sumoylation of transcription factors has generally been correlated with transcriptional repression (9, 10). The specific effects, however, have to be determined experimentally for each case.

In this study, we characterize the modification of PARP1 through SUMO1 and SUMO3. The modification primarily occurs at a lysine residue within the automodification domain of PARP1. The attachment site is close to hotspots of other post-translational modifications of PARP1, such as ADP-ribosylation and acetylation. This proximity led us to investigate a potential crosstalk of these modifications. Sumoylation of PARP1 inhibits its acetylation through p300, and correspondingly, a sumoylation-deficient PARP1 mutant has a higher acetylation status than wild-type PARP1. In addition, a PARP1 sumoylation-deficient cell line exhibits increased transcriptional activity of genes under the control of transcription factor HIF1-α.

MATERIALS AND METHODS

Chemicals and antibodies

Protein G sepharose and glutathione sepharose 4B were purchased from GE Healthcare (Les Ulis, France), ^{32}P-NAD$^+$ and ^{35}S-methionine were from PerkinElmer (Boston, MA, USA). NAD$^+$, trichostatin A (TSA), acetyl-coenzyme A, 3AB, ATP, anti-tubulin, and anti-Flag (M2) were obtained from Sigma-Aldrich (Milan, Italy). Anti-p300 (C20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-HA antibody 16B12 from Covance (Evansville, IL, USA). Anti-myc antibody (9E10) was purchased from Roche (Basel, Switzerland), SUMO2/3 (18H8) was obtained from Cell Signaling (Beverly, MA, USA), and His antibody was from Qiagen (Valencia, Spain). Monoclonal CAIX antibody supernatant from hybridoma was a gift from D. Stiehl (University of Zurich, Zurich, Switzerland). Anti-PARP1 was produced in this laboratory; anti-acetyl-PARP1 was generated in collaboration with the monoclonal antibody core facility at the EMBL Monterotondo (Monterotondo, Italy).

Cell culture and transfection

HEK293T and K562 cells were grown under standard conditions. Transfections were carried out with the calcium phosphate method. Whole-cell extracts were prepared as described previously (20) with 10 mM NEM and/or HDAC inhibitors (2 μM TSA, 5 mM NAM, 1 mM Na-butyrate). Nuclear extracts were prepared as described previously (6).

Plasmids

The baculovirus expression vectors pQE-TriSystem (Qiagen) and BacPak8 (Clontech, Mountain View, CA, USA) were used for the expression of recombinant proteins in Sf21 insect cells, as described previously (21). pcDNA-myc-SUMO1, myc-SUMO3, and myc-Ubc9 expression plasmids were kindly provided by R. T. Hay (University of Dundee, Dundee, UK). PARP1 was cloned into a pCMV-HA vector with *Nhe*I/*Not*I restriction enzymes. pCU vector with Ubc9 was a kind gift from R. Niedenthal (Hannover Medical School, Hannover, Germany). PARP1 was cloned into pCU with *Nhe*I/*Sma*I restriction enzymes, generating a 15-aa linker between PARP1 and Ubc9. pCMV-Flag-p300 was used for expression in mammalian cells. Plasmids for SUMO proteases SENP1-6 were in

pCI-Flag backbone. Short hairpin RNA was cloned and expressed in pSUPER vector.

Cloning, expression, and purification of recombinant proteins

Wild-type hPARP1 (National Center for Biotechnology Information ID: BC037545) was cloned and expressed as amino-terminal HA-tagged and carboxyl-terminal His-tagged protein. HA-PARP1, HA-PARP1 K486R, p300, SUMO1, SUMO3, and Ubc9 proteins were purified by 1-step affinity chromatography using ProBond resin, according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). GST-SUMO3, SUMO3, SENP2 (aa 364-569), and SENP2 (aa 364-569 C548S) were cloned in pGEX-vectors, expressed and purified with glutathione sepharose, according to the manufacturer's recommendations (GE Healthcare). The double-tagged heterodimeric human E1-activating enzyme was expressed from the pGEX-E1H6 vector and purified by sequential GST beads and nickel beads; GST-cleavage was performed through thrombin, and the recombinant protein was loaded and eluted from nickel beads using standard protocols.

In vitro sumoylation assay

The reaction was carried out in standard SUMO reaction buffer (50 mM Tris-HCl, pH 8.0; 50 mM NaCl; 5 mM $MgCl_2$; 10% glycerol; and 0.5 mM DTT). 5 mM ATP was added to start the reaction. Incubation time was 30 min at 30°C, unless otherwise indicated. The final concentration of proteins was 100 nM for SAE1/SAE2, 500 nM Ubc9, 5 μM SUMO1/SUMO3, and 500 nM HA-PARP1.

Purification of sumoylated PARP1

The sumoylation reaction was 15× scaled up, and the incubation time was increased to 120 min at 30°C. Instead of SUMO3, a GST-tagged SUMO3 at a final concentration of 10 μM was used. After sumoylation, the sample was diluted with 2× the volume with SUMO-purification buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; and 1 mM DTT) and bound to glutathione sepharose beads. After 60 min of incubation on rolls at 4°C, the supernatant was washed away with the same buffer, and 2 U of PreScission protease was added to the beads and incubated 16 h at 4°C. The supernatant was used for experiments with sumoylated PARP1.

Desumoylation of PARP1 *in vitro*

Purified sumoylated PARP1 was subjected to active recombinant SENP2 (aa 364-569) or inactive SENP2 (aa 364-569 C548S) treatment in SUMO-purification buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; and 1 mM DTT) for 15 min at 30°C with a concentration of 10 ng SENP2/μl.

^{32}P-NAD automodification

Sumoylated or desumoylated PARP1 in SUMO-purification buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; and 1 mM DTT) was supplemented with 4 mM $MgCl_2$ and 5 pmol of annealed double-stranded oligomer (5′-GGAATTCC-3′). The reaction was started by adding ^{32}P-NAD^+ at a final concentration of 100 nM NAD^+. Automodification was allowed for 5 min at 30°C. Reactions were stopped by the addition of SDS-PAGE-loading buffer and boiling for 5 min at 95°C. Samples were subjected to SDS-PAGE, followed by detection of automodification by autoradiography.

PAR detection by silver staining

Following synthesis of PAR in the presence of 400 μM NAD^+ and 5 pmol *Eco*RI-linker DNA for 20 min, PAR chains were purified and separated by modified DNA-sequencing gel electrophoresis, as described previously (22).

Immunoprecipitation and nickel-NTA pulldown

Sumoylated or desumoylated PARP1 was bound to protein G sepharose beads with anti-HA antibody in SUMO-purification buffer. The beads were washed and adjusted to IP buffer (50 mM Tris-HCL, pH 8.0; 100 mM NaCl; 0.25% Nonidet P-40; and 1 μg/ml protease inhibitors). Recombinant p300 (2 μg) was added to the beads and incubated for 2 h at 4°C on rolls. Washing of the beads with the same buffer removed unbound p300. Immunoprecipitation of nuclear extracts was done with HA antibody with IP-binding buffer (20 mM HEPES, 150 mM NaCl, and 0.25% Nonidet P-40; 1 μg/ml), protease inhibitors, and HDAC inhibitors (2 μM TSA, 5 mM NAM, 1 mM Na-butyrate). The salt concentration was increased with 50 mM KCl for washing steps. Elution of bound proteins was done with SDS-PAGE loading buffer and boiled for 5 min at 95°C. Nickel-NTA pulldown was done as described previously (23).

HAT Assay

Sumoylated or desumoylated PARP1 was subjected to *in vitro* acetylation assay with recombinant p300 as described elsewhere (24).

Knockdown and complementation of PARP1 in K562 cells

Generation of viruses and transduction of cells was done as described earlier (25). shRNA was cloned into pRDI vector and transduced to K562 cells. The short hairpin RNA was designed against 5′UTR region of PARP1 mRNA. Transduced cells were selected through puromycine resistance gene. Complementation of cells was done with pRRL-myc-PARP1 vectors containing a blasticidine resistance marker and subsequently selected with this antibiotic.

RNA preparation

Total RNA was isolated from 3 biological replicates of complemented K562 cells with the Total RNA Isolation kit (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription was achieved with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

Quantitative PCR

Total reverse-transcribed cDNA from untreated or treated K562 cells was used for q-PCR with primers against carbonic anhydrase IX, LOXL2, and Pdk1. Amplification products were analyzed by SYBR Green (Quantace, London, UK), and ribosomal protein L28 was used to normalize for differences in RNA input. Rotor-Gene3000A (Qiagen, Basel, Switzerland) was used to perform the real-time PCR reactions.

RESULTS

PARP1 is sumoylated *in vivo*

Because PARP1 was identified as a SUMO modification target in proteomic studies, we aimed to confirm that PARP1 is indeed sumoylated *in vivo*. HA-tagged PARP1 was coexpressed with myc-tagged SUMO1 or SUMO3 in HEK293T cells, and extracts were analyzed by Western blot. Ectopic expression of SUMO1 or SUMO3 *per se* induced the modification of a multitude of proteins (**Fig. 1*A***, bottom). Expression of SUMO induced a higher molecular form of PARP1 (depicted as Su-PARP1), which was more prominent in the presence of SUMO3 as compared to SUMO1, suggesting that PARP1 is preferentially conjugated with SUMO3 (Fig. 1*A*). Expressing His-tagged SUMO, we could enrich an anti-PARP1-reactive species on Ni-NTA beads, thus validating that the higher molecular form corresponds, indeed, to a covalent SUMO-PARP1 conjugate (Fig. 1*B*). Only one distinctive band of sumoylated PARP1 was detected, suggesting that PARP1 is monosumoylated at a single lysine residue under the tested conditions. Similar results were obtained when PARP1 was expressed as fusion protein with Ubc9/E2 conjugation protein, although the overall modification rate was clearly enhanced (Supplemental Fig. 1*A*). Mutation of the catalytic cysteine of the fused Ubc9 resulted in a strong reduction of the modification, indicating that the Ubc9 fused to PARP1 catalyzes the sumoylation of PARP1 (Supplemental Fig. 1*B*). Immunoprecipitation of this fusion protein in extracts of cells expressing myc-tagged SUMO3 and subsequent Western blot analysis using an anti-myc antibody revealed SUMO moieties on PARP1, providing additional evidence for covalent modification of PARP1 with SUMO (Supplemental Fig. 1*C*). To test whether PARP1 would bind to SUMO noncovalently *via* a SUMO-interacting motif (SIM), GST pulldowns were performed with conjugation-deficient SUMO1-4 and RelA/p65 as a positive control (Supplemental Fig. 2*A–C*). Although PARP1 was able to interact with RelA/p65, no interaction was detectable with GST or all tested SUMOs. Thus, we conclude that PARP1 is covalently modified by SUMO.

SENP1 and SENP3 are able to desumoylate PARP1

SUMO proteases are known to reverse sumoylation of proteins. To test whether SUMO proteases act on

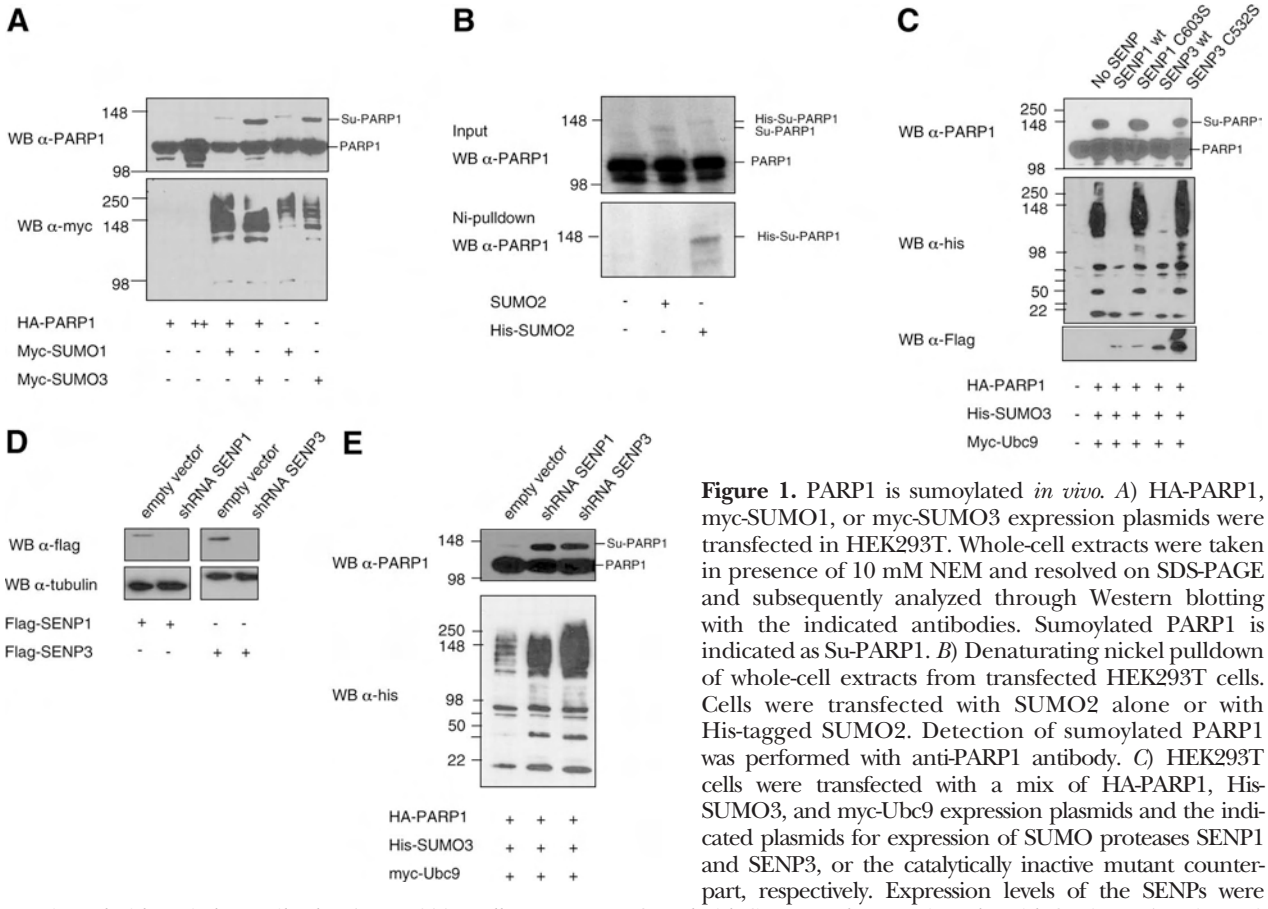


Figure 1. PARP1 is sumoylated *in vivo*. *A*) HA-PARP1, myc-SUMO1, or myc-SUMO3 expression plasmids were transfected in HEK293T. Whole-cell extracts were taken in presence of 10 mM NEM and resolved on SDS-PAGE and subsequently analyzed through Western blotting with the indicated antibodies. Sumoylated PARP1 is indicated as Su-PARP1. *B*) Denaturating nickel pulldown of whole-cell extracts from transfected HEK293T cells. Cells were transfected with SUMO2 alone or with His-tagged SUMO2. Detection of sumoylated PARP1 was performed with anti-PARP1 antibody. *C*) HEK293T cells were transfected with a mix of HA-PARP1, His-SUMO3, and myc-Ubc9 expression plasmids and the indicated plasmids for expression of SUMO proteases SENP1 and SENP3, or the catalytically inactive mutant counterpart, respectively. Expression levels of the SENPs were monitored with anti-Flag antibody. *D*) HEK293T cells were cotransfected with flag-tagged expression plasmids for SENP1 or SENP3 and pSUPER vector with shRNAs against SENPs. Knockdown efficiency after 28-h expression was examined with anti-Flag antibody. *E*) Knockdown of SENP1 and SENP3 was achieved as in *D*, but in addition, a mix of HA-PARP1, His-SUMO3, and myc-Ubc9 was cotransfected in HEK293T cells. Cell extracts were prepared and examined with the anti-PARP1 and anti-His antibodies.

SUMO-modified PARP1, we coexpressed wild-type or catalytically inactive SENP1 and SENP3 with PARP1 and SUMO3 in HEK293T cells (Fig. 1*C*). This showed that coexpression of catalytically active SENP1 and SENP3 deconjugated SUMO3 from PARP1 (Fig. 1*C*). Correspondingly, knockdown of SENP1 and SENP3 with transiently transfected shRNAs (Fig. 1*D*) resulted in the accumulation of sumoylated PARP1, as compared to the control (Fig. 1*E*), indicating that SENP1 and SENP3 can act on PARP1-SUMO conjugates at physiological expression levels. Taken together, our results illustrate that PARP1 is preferably modified by SUMO3 and desumoylated by the isopeptidases SENP1 and SENP3. Furthermore, sumoylation of PARP1 seems thus to be a transient and reversible modification.

PARP1 is sumoylated at K486 *in vitro* and *in vivo*

The consensus sumoylation site sequence is ΨKxE/D (8). As determined by the SUMOsp analysis program (http://sumosp.biocuckoo.org), the highest score matched to lysine 486 in human PARP1 (**Fig. 2*A***), which is located in proximity to previously described sites of acetylation and ADP ribosylation. To confirm PARP1 sumoylation *in vitro* and to map the modification site, we established an *in vitro* sumoylation system reconstituted with recombinant human E1 (SAE1/SAE2 heterodimer), E2 (Ubc9), wild-type SUMO1, or SUMO3 and wild-type PARP1 (Fig. 2*B*). PARP1 sumoylation was efficiently reconstituted *in vitro* with purified proteins: reactions containing all components produced slower migrating PARP1 forms, consistent with conjugated SUMO moieties. Attachment of a single moiety was detected with low E2 concentrations (running at ~140 kDa), whereas multiple SUMO moieties were attached only at higher E2 concentrations (Fig. 2*B*). One additional band between 120 and 140 kDa was observed at elevated Ubc9 concentrations and likely represents the modification of a degradation product of PARP1 by SUMO3. To test for putative sumoylation sites in PARP1, lysine 486 of PARP1 was substituted with arginine (PARP1 K486R) and analyzed *in vitro*. Substitution did completely prevent the sumoylation of PARP1 *in vitro* with SUMO1, SUMO3, or GST-tagged SUMO3, as monitored by Western blot analysis (Fig. 2*C, D*), indicating that K486 is the major SUMO acceptor site of PARP1.

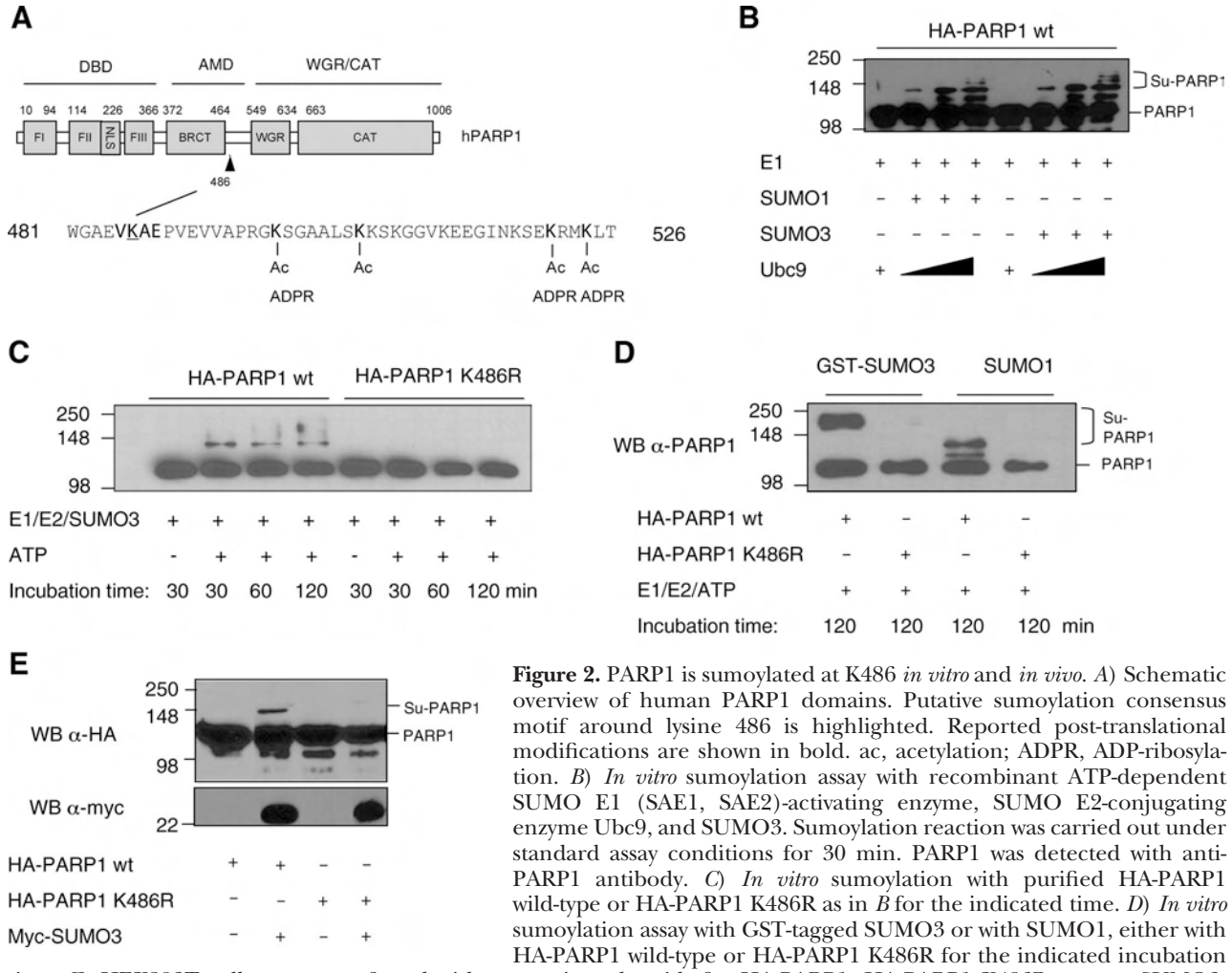


Figure 2. PARP1 is sumoylated at K486 *in vitro* and *in vivo*. *A*) Schematic overview of human PARP1 domains. Putative sumoylation consensus motif around lysine 486 is highlighted. Reported post-translational modifications are shown in bold. ac, acetylation; ADPR, ADP-ribosylation. *B*) *In vitro* sumoylation assay with recombinant ATP-dependent SUMO E1 (SAE1, SAE2)-activating enzyme, SUMO E2-conjugating enzyme Ubc9, and SUMO3. Sumoylation reaction was carried out under standard assay conditions for 30 min. PARP1 was detected with anti-PARP1 antibody. *C*) *In vitro* sumoylation with purified HA-PARP1 wild-type or HA-PARP1 K486R as in *B* for the indicated time. *D*) *In vitro* sumoylation assay with GST-tagged SUMO3 or with SUMO1, either with HA-PARP1 wild-type or HA-PARP1 K486R for the indicated incubation time. *E*) HEK293T cells were transfected with expression plasmids for HA-PARP1, HA-PARP1 K486R, or myc-SUMO3, respectively. Whole-cell extracts were analyzed with anti-HA antibody and anti-myc antibody. Saturated levels of unbound myc-SUMO3 were detected in the control Western blot.

To verify sumoylation of PARP1 at K486 *in vivo*, we coexpressed wild-type or the K486R mutant of PARP1 with myc-tagged SUMO3. Sumoylation of wild-type PARP1 could be detected but not of the K486R mutant (Fig. 2*E*), confirming K486 as the main sumoylated residue *in vivo*.

Sumoylation of PARP1 does not affect its ADP ribosylation activity

To explore a potential interplay of PARP1 sumoylation with PARP1 function, we modified the established *in vitro* sumoylation system to purify sumoylated PARP1. A large-scale sumoylation reaction was performed with GST-tagged SUMO3, E1–E2 enzymes, and HA-PARP1, followed by GST affinity purification and subsequent protease digestion to remove the GST tag and to purify Su-PARP1, specifically modified at K486 (**Fig. 3*A***). On purification, only Su-PARP1 could be detected, indicating that no unmodified PARP1 was in the purified complex (Fig. 3*B*). Because PARP1 was described to form a homodimer, this result suggests that both subunits are equally accessible for SUMO-conjugation. PARP1 was also efficiently sumoylated in the presence of double-stranded DNA ends, suggesting that binding of PARP1 to DNA does not affect its sumoylation *in vitro* (Supplemental Fig. 3*A*). Similarly, Su-PARP1 was still able to bind specifically to DNA fragments that mimic damaged DNA (Supplemental Fig. 3*B*).

To determine whether sumoylation regulates the intrinsic ADP-ribosylation activity of PARP1, mono-ADP-ribosylation of purified Su-PARP1 was measured using an *in vitro* ADP-ribosylation assay in the presence of 100 nM ^{32}P-NAD$^+$. Sumoylation of PARP1 still allows its mono-ADP-ribosylation activity (Fig. 3*B*, lane 1). To compare the extent of ADP ribosylation, Su-PARP1 was either desumoylated by recombinant SENP2 (aa 364-569) before or after ADP ribosylation took place (Fig. 3*B*, lanes 2 and 3). Quantification of the detected radioactivity confirmed that both proteins were modified to the same extent. Similar experiments were repeated with 400 μM NAD$^+$, a concentration that allows detection of poly(ADP-ribosyl)ation of PARP1. PAR polymers synthesized by Su-PARP1 and desumoylated PARP1 were isolated and analyzed with silver-stained PAGE (Fig. 3*C*). Neither the amount nor the distribution of freshly synthesized PAR was altered by sumoylated PARP1, indicating that SUMO modification neither alters the ability of PARP1 to initiate nor to extend PAR synthesis. In addition, overexpression of SUMO3 in HEK293T cells *per se* did not stimulate PAR formation (Supplemental Fig. 4*A*), although PARP1 is sumoylated under these conditions (see Fig. 1*A*, last lane). Furthermore, H_2O_2-treated cells showed PAR formation (Supplemental Fig. 4*B*), which was independent of SUMO3 levels, indicating that SUMO modification of PARP1 does not enhance its poly(ADP-ribosyl)ation activity. To test the possibility of differential localization of PARP1 upon sumoylation, we overex-

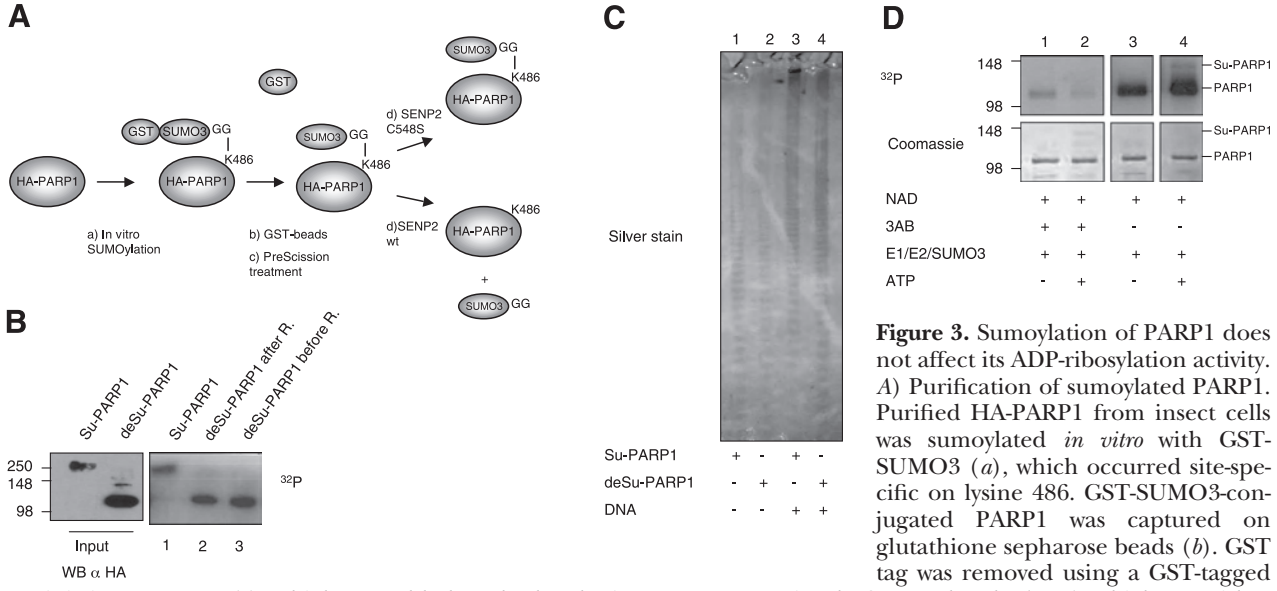


Figure 3. Sumoylation of PARP1 does not affect its ADP-ribosylation activity. *A*) Purification of sumoylated PARP1. Purified HA-PARP1 from insect cells was sumoylated *in vitro* with GST-SUMO3 (*a*), which occurred site-specific on lysine 486. GST-SUMO3-conjugated PARP1 was captured on glutathione sepharose beads (*b*). GST tag was removed using a GST-tagged PreScission protease (*c*), which was added to the beads. Supernatant consisted of sumoylated PARP1, which was either incubated with active recombinant SENP2 fragment (aa 364-569) or the inactive mutant SENP2 C548S. *B*) Sumoylated PARP1 (lanes 1 and 2) or desumoylated PARP1 (lane 3) was incubated with 100 nM radiolabeled ^{32}P-NAD$^+$ and 5 pmol *Eco*RI-linker DNA. Reaction was stopped with PARP-inhibitor 3AB, and active SENP2 was added to deconjugate SUMO3 from PARP1 (lane 2), thus generating free PARP1. Mono(ADP-ribosyl)ation was monitored with autoradiography. *C*) Silver stain of isolated PAR generated by sumoylated PARP1 (lanes 1 and 3) or by desumoylated PARP1 (lanes 2 and 4). Reaction was carried out in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of *Eco*RI-linker DNA at 400 μM NAD$^+$. *D*) Mono(ADP-ribosyl)ation of PARP1 in the presence or absence of PARP-inhibitor 3AB with 100 nM NAD$^+$. Each sample was supplemented with E1, E2, and SUMO3 proteins. Reaction was stopped after a 5-min incubation time with 3AB (lanes 3 and 4), and ATP was added (lanes 2 and 4). After the sumoylation reaction, proteins were separated by SDS-PAGE and analyzed by autoradiography.

pressed SUMO3 in cells and monitored PARP1 localization by immunofluorescence. However, we did not observe differential localization of PARP1 within the nucleus upon ectopic SUMO3 expression (Supplemental Fig. 4*C*).

We recently reported that PARP1 is auto-ADP-ribosylated at several lysines adjacent to the identified sumoylation site (26). To exclude that ADP-ribosylation would affect sumoylation, we mono-ADP-ribosylated PARP1 *in vitro* with radioactive NAD^+ and subsequently sumoylated the labeled PARP1 fraction (Fig. 3*D*). PARP1 was sumoylated in an ATP-dependent manner independent of its ADP-ribosylation. Consistently, *in vivo* treatment of cells with the PARP inhibitor 3-amino-benzamide (3AB) did not affect sumoylation (data not shown). Taken together, this suggests that although the sumoylated and ADP-ribosylated lysines are rather close within the PARP1 amino acid sequence, their modifications do not interfere with each other.

Sumoylation counteracts p300-induced acetylation of PARP1

As p300 is critical for PARP1 transcriptional coactivation and acetylates PARP1 at distinct lysines (6), we first examined whether acetylated PARP1 would still be sumoylated *in vitro*. Acetylation of PARP1 was monitored with a specific anti-acetyl PARP1 (E4) antibody (Supplemental Fig. 5*A*), while sumoylation was assessed by the migration difference between unmodified PARP1 and Su-PARP1. Acetylation with the indicated control and subsequent addition of sumoylation enzymes, followed by the sumoylation reaction, revealed that similar to the mono-ADP-ribosylated PARP1, acetylated PARP1 could also be efficiently modified with SUMO (**Fig. 4*A***, lane 4).

Moreover, we tested whether sumoylation of PARP1 would affect acetylation. Purified Su-PARP1 or desumoylated PARP1 by recombinant SENP2 was both incubated with p300 and acetyl-CoA *in vitro*. Western blot analysis using the specific anti-acetyl PARP1 (E4) antibody revealed that PARP1 is acetylated only when PARP1 was desumoylated prior to acetylation (Fig. 4*B*, lane 2), suggesting that the SUMO-modification inhibits p300-mediated PARP1 acetylation. To substantiate this, we examined protein interactions with p300, PARP1, or Su-PARP1. p300 could interact efficiently with PARP1 but not with Su-PARP1, as demonstrated by coimmunoprecipitation of p300 (Fig. 4*C*). Thus, the absence of detectable acetylation of Su-PARP1 (see Fig. 4*B*) suggests that SUMO modification at K486 prevents p300-mediated acetylation of PARP1, likely because of steric hindrance of the bulky SUMO conjugate blocking p300 binding and acetylation at the adjacent lysine residues. To explore whether the inhibitory effect of PARP1 sumoylation on acetylation is also observed *in vivo*, we coexpressed wild-type PARP1 or the sumoylation-deficient PARP1 mutant (K486R) with p300 and monitored acetylation with the E4 antibody on Western blots. This revealed lower levels of acetylation for the wild-type PARP1 compared to the sumoylation-defi-

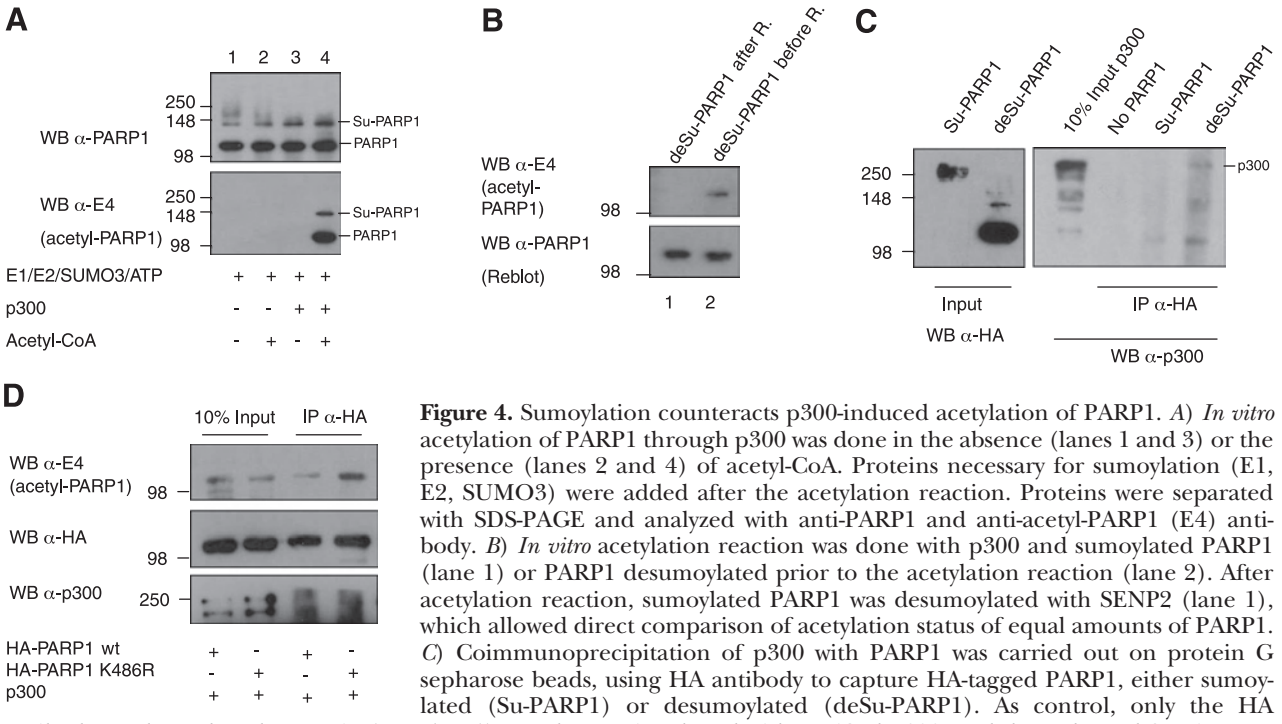


Figure 4. Sumoylation counteracts p300-induced acetylation of PARP1. *A*) *In vitro* acetylation of PARP1 through p300 was done in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of acetyl-CoA. Proteins necessary for sumoylation (E1, E2, SUMO3) were added after the acetylation reaction. Proteins were separated with SDS-PAGE and analyzed with anti-PARP1 and anti-acetyl-PARP1 (E4) antibody. *B*) *In vitro* acetylation reaction was done with p300 and sumoylated PARP1 (lane 1) or PARP1 desumoylated prior to the acetylation reaction (lane 2). After acetylation reaction, sumoylated PARP1 was desumoylated with SENP2 (lane 1), which allowed direct comparison of acetylation status of equal amounts of PARP1. *C*) Coimmunoprecipitation of p300 with PARP1 was carried out on protein G sepharose beads, using HA antibody to capture HA-tagged PARP1, either sumoylated (Su-PARP1) or desumoylated (deSu-PARP1). As control, only the HA antibody was bound to the matrix (no PARP1). Beads were incubated with purified p300, and the unbound fraction was removed by extensive washing. *D*) HEK293T cells were transfected with either HA-PARP1 wt or HA-PARP1 K486R mutant along with p300 expression plasmid. Cells were incubated with HDAC inhibitors 2 h prior to lysis, and HDAC inhibitors were present at all steps of manipulation. Nuclear extracts were taken and subjected to immunoprecipitation using an HA antibody. After SDS-PAGE, proteins were detected by anti-acetyl-PARP1 (E4), anti-HA, or anti-p300 antibody.

cient PARP1 mutant (Fig. 4*D*). Together, these results provide evidence for a crosstalk between these modifications.

The sumoylation-deficient K486R PARP1 mutant exhibits higher coactivator function compared to wild-type PARP1

To explore a possible mechanism by which sumoylation affects PARP1-dependent transcriptional coactivator function *in vivo*, we first knocked down endogenous PARP1 protein levels in K562 cells with an shRNA approach directed against the untranslated region of PARP1's mRNA and subsequently complemented these cells with wild-type or sumoylation-deficient K486R PARP1 mutant (**Fig. 5*A, B***). The expression levels of the complemented cells were comparable to the endogenous wild-type counterpart. Hypoxia treatment of these cells for 28 h and subsequent profiling of the gene expression of defined hypoxia-inducible genes revealed that certain genes, such as CAIX, LOXL2 or Pdk1, are dependent on PARP1, but only a subset was affected by the sumoylation-deficient K486R mutation (Fig. 5*C*). Similar results were obtained when PARP1$^{-/-}$ mouse lung fibroblasts were complemented with wild-type or sumoylation-deficient K486R PARP1 mutant and stimulated by the hypoxia-mimicking drug ciclopiroxolamine (Supplemental Fig. 5*B*). Sumoylation-deficient K486R PARP1 mutant not only enhanced CAIX mRNA levels in K562 cells, but also CAIX protein levels *in vivo* (Fig. 5*D*). Furthermore, hypoxia treatment of K562 cells very strongly correlated with protein sumoylation and enhanced SUMO modification of PARP1 in HEK293T cells (Supplemental Fig. 5*C, D*). Thus, we conclude that sumoylation of PARP1 reduces its coactivator activity and thus regulates gene expression *in vivo*.

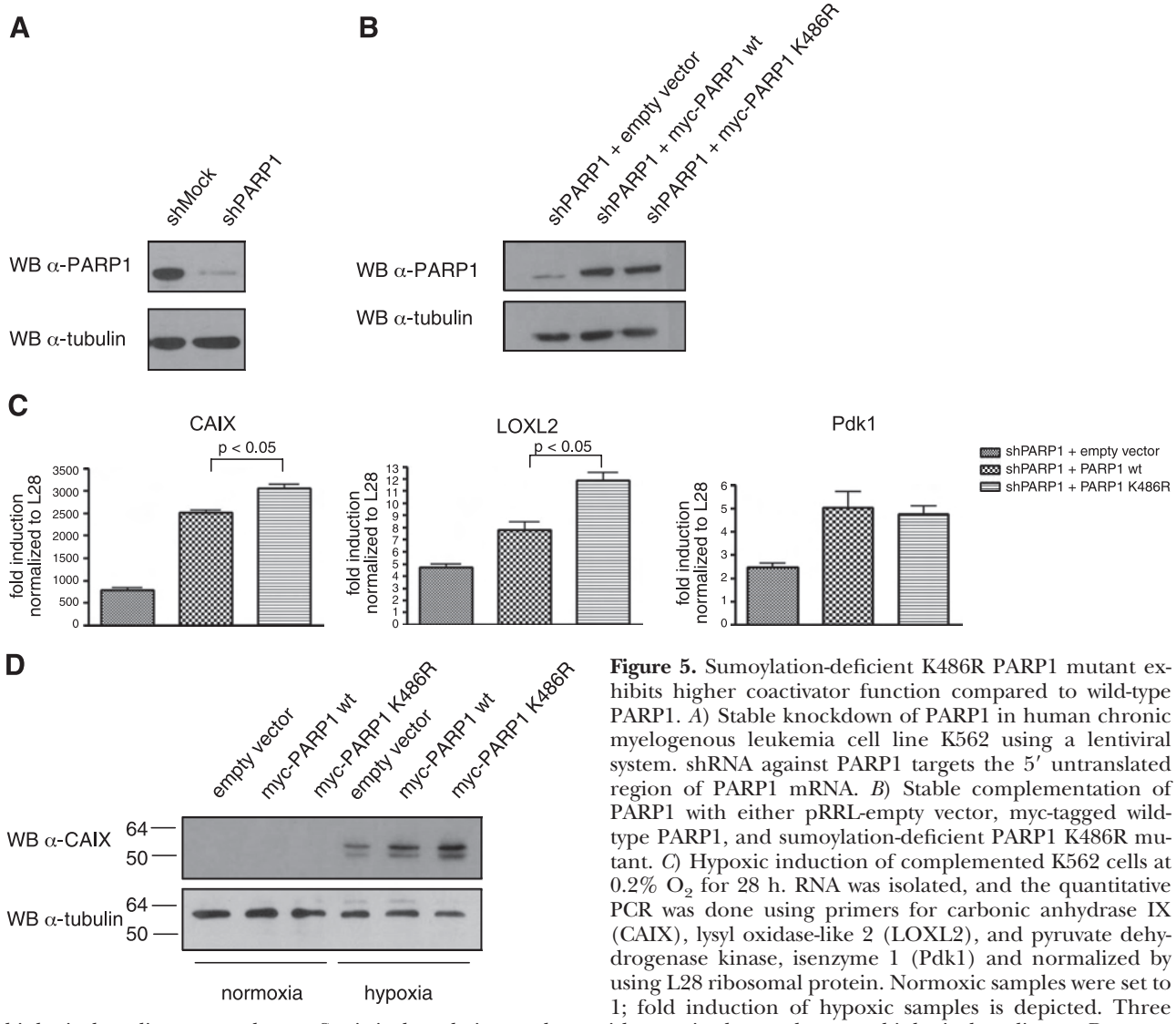


Figure 5. Sumoylation-deficient K486R PARP1 mutant exhibits higher coactivator function compared to wild-type PARP1. *A*) Stable knockdown of PARP1 in human chronic myelogenous leukemia cell line K562 using a lentiviral system. shRNA against PARP1 targets the 5′ untranslated region of PARP1 mRNA. *B*) Stable complementation of PARP1 with either pRRL-empty vector, myc-tagged wild-type PARP1, and sumoylation-deficient PARP1 K486R mutant. *C*) Hypoxic induction of complemented K562 cells at 0.2% O_2 for 28 h. RNA was isolated, and the quantitative PCR was done using primers for carbonic anhydrase IX (CAIX), lysyl oxidase-like 2 (LOXL2), and pyruvate dehydrogenase kinase, isenzyme 1 (Pdk1) and normalized by using L28 ribosomal protein. Normoxic samples were set to 1; fold induction of hypoxic samples is depicted. Three biological replicates are shown. Statistical analysis was done with unpaired *t* test between biological replicates. Data are represented as means ± SE. *D*) Complemented K562 cells were exposed for 29 h to 0.2% O_2 hypoxia. Whole-cell extracts were prepared and Western blotted with monoclonal anti-CAIX antibody and anti-tubulin antibody.

DISCUSSION

The aim of this study was to characterize and investigate the role of PARP1 sumoylation in the cellular context. We provide biochemical and cellular evidence for SUMO modification of PARP at lysine 486 within its automodification domain. Mutation of K486 enhances the transcriptional activity of PARP1, suggesting that sumoylation restrains its transcriptional activity.

PARP1 is covalently sumoylated

Noncovalent interactions of proteins can occur through SUMO interaction motifs (SIMs) (27). Proteins like the DNA repair enzyme TDG and the tumor suppressor PML were shown to interact with SUMOs *via* SIMs, and such interactions were associated with important biological activities (28–30). Although PARP1 exhibits several putative SIMs, we did not observe any direct noncovalent interaction of PARP1 with SUMOs, indicating that the interaction of PARP1 with SUMO is exclusively covalent. Pulldown experiments of sumoylated PARP1 under denaturating conditions and site-directed mutagenesis revealed that sumoylation of PARP1 is indeed a covalent and site-specific modification. A possible involvement of SUMO E3 ligases for the sumoylation of PARP1 needs further investigations. Although PIAS family members are attractive candidates, overexpression of different PIAS proteins did not enhance PARP1 sumoylation (data not shown).

Notably, we observed only monosumoylation of PARP1 *in vivo*, but do not exclude that under specific conditions, PARP1 may also be polysumoylated. In support of this idea, heat shock has been reported to induce a pattern of PARP1 sumoylation, which would be consistent with polysumoylation (18). Understanding the balance between monosumoylation and polysumoylation of PARP1, as well as their functional differences will remain an exciting issue.

Crosstalk between sumoylation and other post-translational modifications

The crosstalk of post-translational modification systems is an emerging concept (31). Sumoylation of target proteins can be regulated through crosstalks with other post-translational modification events. Phosphorylation, for instance, was shown to regulate SUMO conjugation through a highly conserved motif, which is called phosphorylation-dependent sumoylation motif (PDSM) (32). The PDSM motif, which contains a SUMO consensus site and an adjacent proline-directed phosphorylation site (ΨKxExxSP, where Ψ represents large hydrophobic residue and x is any amino acid), regulates phosphorylation-dependent sumoylation of multiple transcription factors (33–35). Lysine residues are targeted by several other post-translational modifications, including ubiquitination, acetylation, methylation, and ADP-ribosylation. It has been documented that SUMO conjugation can occur on the same lysine residue as ubiquitination or acetylation in some proteins. For example, the competition between sumoylation and ubiquitination of the same lysine regulates the stability of IκBα (36), whereas in other cases, sumoylation acts as a recognition signal for a ubiquitin ligase (37). An interplay between sumoylation and acetylation has been observed in the regulation of proteins, such as MEF2, the core histones, and hypermethylation in cancer 1 (HIC1) (38, 39). In the case of MEF2, the sumoylation-acetylation switch is regulated by phosphorylation (40). These studies demonstrate the importance of signaling crosstalk in the regulation of protein sumoylation.

Mechanisms of SUMO-mediated repression of PARP1 coactivator function

First, sumoylation may directly affect PARP1's binding to DNA by promoting its dissociation from specific chromatin regions. This possibility seems unlikely, since sumoylation of PARP1 did not alter its ability to recognize and bind damaged DNA *in vitro*. Second, SUMO modification could also affect enzymatic activities of PARP1, which is important for gene expression. Also, this seems unlikely, since we have shown that SUMO modification of PARP1 does not interfere with DNA-dependent ADP-ribosylation activity *in vitro*. In addition, increased SUMO3 levels do not correspond to elevated poly(ADP-ribosyl)ation in cells on hydrogen peroxide-induced DNA damage, suggesting that sumoylation of PARP1 does not have a stimulatory effect on its enzymatic activity. However, it was shown that poly(ADP-ribosyl)ation is not required for NF-κB-dependent gene expression (41). Neither the enzymatic activity of PARP1 nor its binding to DNA was required for full activation of NF-κB in response to various stimuli *in vivo* when tested on transiently transfected reporter plasmids (21, 42). Consistently, the enzymatic activity of PARP1 was not required for full transcriptional activation of NF-κB in the presence of the histone acetyltransferase p300 (6). Because sumoylation of PARP1 inhibits its acetylation at adjacent lysine residues and because these residues are also targets of ADP-ribosylation, a potential acetylation-ADP-ribosylation switch, which is controlled through sumoylation of PARP1, is very likely. Third, the SUMO modification could promote or inhibit protein-protein interactions through protein complex formation. This scenario seems to be the most relevant for PARP1, since the interaction of PARP1 with p300 and subsequent PARP1 acetylation was impaired after sumoylation of PARP1 at K486. This lysine residue lies within the domain of PARP1, which contributes to most protein-protein interactions such as XRCC1 (6). However, we did not observe a general SUMO-dependent inhibition of protein interactions in this region since HIF1-α and XRCC1 binding does not seem to be affected by sumoylation of PARP1 (Supplemental Fig. 5*E* and unpublished results). In addition to the inhibition of p300

binding, SUMO modification of PARP1 may facilitate the recruitment of a transcriptional corepressor. Currently, several chromatin-modifying enzymes and chromatin-binding proteins have been implicated as effectors of SUMO-mediated repression. For example, SUMO modification of the transcription factor Elk-1 promotes recruitment of HDAC2, associated with histone deacetylation and transcriptional repression of the c-fos promoter (43). Very recently, CoREST1 and Mi2 were identified as SUMO-dependent corepressors, and evidence was provided that CoREST1 binds directly and noncovalently to SUMO2/3, but not to SUMO1 (44, 45). Notably, the aforementioned interaction of PARP1 with PIAS family members could contribute to gene silencing.

Desumoylation of PARP1 by SENP1 and SENP3

We observed that SENP1 and SENP3 are able to catalyze PARP1's SUMO deconjugation. The nucleoplasmic SENP1 relieves SUMO-dependent repression of Ets1, c-Jun, and the androgen receptor, the latter effect being through desumoylation of histone deacetylase 1 (46). Recent data also implicate the nucleolus in dynamic cycles of sumoylation and desumoylation. For example, nucleolar SENP3 is able to catalyze desumoylation of various proteins in this compartment, with specificity to SUMO2/3 (15, 46). In addition, it seems that SENPs regulate SUMO paralog preference of substrate proteins by deconjugation of specific SUMOs, as shown for RanGAP1 (47). This could also explain the higher steady-state level of SUMO3-modified than SUMO1-modified PARP1.

Only a subset of PARP1-dependent genes are affected by sumoylation

Analyses of the role of SUMO in transcriptional regulation have mainly relied on the use of protein overexpession and transiently transfected reporter genes, which may not give a true reflection of the physiological situation. Therefore, we have established a system where we complement cells depleted from endogenous PARP1 with sumoylation deficient PARP1 or wild-type PARP1 and analyzed the expression of endogenous target genes. Known HIF1-α dependent genes with a high induction upon hypoxia were tested. Of these, CAIX and LOXL2 showed increased transcript levels in sumoylation deficient K486R mutant cell line, whereas other genes were solely dependent on PARP1, but not on its sumoylation. Consistent with the qPCR data, the expression levels of CAIX were increased in cells expressing sumoylation-deficient PARP1. Previous studies on PARP1's coactivator function revealed that this function is heavily dependent on its acetylation through p300. Here, we showed that acetylation is abrogated if the SUMO moiety is present on PARP1. Consistently, the sumoylation-deficient mutant showed a higher acetylation status, which corresponded to higher gene expression status for some genes. Collectively, these data support the mechanistic studies performed *in vitro*, unraveling an important role of sumoylation in regulating PARP1-dependent transcriptional coactivation through regulation of its acetylation. It is largely accepted that post-translational modifications fine tune and regulate the requirement of certain transcriptional cofactors for gene expression by transcription factors and might thus influence only a subset of genes (48).

Hypoxia-induced gene expression is affected by PARP1 sumoylation

The role of sumoylation in the regulation of hypoxia-induced gene expression and HIF-1α stability is controversial (49). Hypoxia can induce the expression of SUMO1 (50) and an RWD-containing sumoylation enhancer (RSUME) that functions as a promoter of protein sumoylation (51). RSUME expression is induced by hypoxia, which leads to the enhanced sumoylation and stabilization of HIF-1α. Alternatively, a recent study indicates that the hypoxia-induced sumoylation of HIF-1α targets HIF-1α for degradation through the von Hippel–Lindau (VHL) protein-mediated ubiquitin-proteasome pathway (37). Clearly, further studies are needed to clarify these controversial findings on the role of sumoylation in the regulation of HIF-1α stability during hypoxia. In this study, we have investigated the regulation of the known HIF1-α-dependent genes CAIX and LOXL2 and provide novel insights to understand the complex transcriptional regulation of these emerging tumor markers. The expression of these genes is restrained through SUMO modification of the transcriptional coactivator PARP1, indicating that sumoylation of PARP1 dampens HIF1-α signaling for these genes. Thus, this pathway may have an important regulatory role in the regulation of intracellular pH and hypoxia-induced metastasis (52, 53). Moreover, it remains to be elucidated whether sumoylation of PARP1 is also affecting transcription mediated by other transcription factors and whether SUMO modification is associated with the role of PARP1 in several pathophysiological disease models. Further studies of the sumoylation of PARP1 will determine the role of SUMO modification/deconjugation in these pathological states. FJ

We are grateful to L. Sistonen and H. A. Blomster (University of Turku, Turku, Finland) for support and scientific insight. We thank G. Poirier and D. Gauvin (Laval University Medical Research Center, Laval University, Quebec, QC, Canada), D. Stiehl and R. Wenger (University of Zurich, Zurich, Switzerland), S. Pastorekova (Slovak Academy of Sciences, Bratislava, Slovak Republic), R. T. Hay (University of Dundee, Dundee, UK), and R. Niedenthal (Hannover Medical School, Hannover, Germany) for generously providing reagents. We are grateful to T. Valovka (University of Innsbruck, Innsbruck, Austria) and P. O. Hassa, M. Fey, and other members of the Institute of Veterinary Biochemistry and Molecular Biology (University of Zurich) for their critical and constructive comments on the manuscript. This work was

supported in part by the Deutsche Forschungsgemeinschaft (to S. Müller), the Swiss National Science Foundation (grants 31-109315.05 and 31-122421/1 to M.O.H.), and the Kanton of Zurich (to M.O.H.).

REFERENCES

1. Hassa, P. O., Haenni, S. S., Elser, M., and Hottiger, M. O. (2006) Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol. Mol. Biol. Rev.* **70,** 789–829
2. D'Amours, D., Desnoyers, S., D'Silva, I., and Poirier, G. G. (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem. J.* **342,** 249–268
3. Kim, M. Y., Zhang, T., and Kraus, W. L. (2005) Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. *Genes Dev.* **19,** 1951–1967
4. Kraus, W. L. (2008) Transcriptional control by PARP-1: chromatin modulation, enhancer-binding, coregulation, and insulation. *Curr. Opin. Cell Biol.* **20,** 294–302
5. Altmeyer, M., Messner, S., Hassa, P. O., Fey, M., and Hottiger, M. O. (2009) Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites. *Nucleic Acids Res.* **37,** 3723–3738
6. Hassa, P. O., Haenni, S. S., Buerki, C., Meier, N. I., Lane, W. S., Owen, H., Gersbach, M., Imhof, R., and Hottiger, M. O. (2005) Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-κB-dependent transcription. *J. Biol. Chem.* **280,** 40,450–40,464
7. Elser, M., Borsig, L., Hassa, P. O., Erener, S., Messner, S., Valovka, T., Keller, S., Gassmann, M., and Hottiger, M. O. (2008) Poly(ADP-ribose) polymerase 1 promotes tumor cell survival by coactivating hypoxia-inducible factor-1-dependent gene expression. *Mol. Cancer Res.* **6,** 282–290
8. Johnson, E. S. (2004) Protein modification by SUMO. *Annu. Rev. Biochem.* **73,** 355–382
9. Gill, G. (2004) SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev.* **18,** 2046–2059
10. Hay, R. T. (2005) SUMO: a history of modification. *Mol. Cell.* **18,** 1–12
11. Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naismith, J. H., and Hay, R. T. (2001) Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J. Biol. Chem.* **276,** 35,368–35,374
12. Saitoh, H., and Hinchey, J. (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J. Biol. Chem.* **275,** 6252–6258
13. Ayaydin, F., and Dasso, M. (2004) Distinct in vivo dynamics of vertebrate SUMO paralogues. *Mol. Biol. Cell* **15,** 5208–5218
14. Vertegaal, A. C., Andersen, J. S., Ogg, S. C., Hay, R. T., Mann, M., and Lamond, A. I. (2006) Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics. *Mol. Cell. Proteomics* **5,** 2298–2310
15. Mukhopadhyay, D., and Dasso, M. (2007) Modification in reverse: the SUMO proteases. *Trends Biochem. Sci.* **32,** 286–295
16. Vertegaal, A. C., Ogg, S. C., Jaffray, E., Rodriguez, M. S., Hay, R. T., Andersen, J. S., Mann, M., and Lamond, A. I. (2004) A proteomic study of SUMO-2 target proteins. *J. Biol. Chem.* **279,** 33,791–33,798
17. Rosas-Acosta, G., Russell, W. K., Deyrieux, A., Russell, D. H., and Wilson, V. G. (2005) A universal strategy for proteomic studies of SUMO and other ubiquitin-like modifiers. *Mol. Cell. Proteomics* **4,** 56–72
18. Blomster, H. A., Hietakangas, V., Wu, J., Kouvonen, P., Hautaniemi, S., and Sistonen, L. (2009) Novel proteomics strategy brings insight into the prevalence of SUMO-2 target sites. *Mol. Cell. Proteomics* **8,** 1382–1390
19. Girdwood, D. W., Tatham, M. H., and Hay, R. T. (2004) SUMO and transcriptional regulation. *Semin. Cell. Dev. Biol.* **15,** 201–210
20. Dadke, S., Cotteret, S., Yip, S. C., Jaffer, Z. M., Haj, F., Ivanov, A., Rauscher, F., 3rd, Shuai, K., Ng, T., Neel, B. G., and Chernoff, J. (2007) Regulation of protein tyrosine phosphatase 1B by sumoylation. *Nat. Cell Biol.* **9,** 80–85
21. Hassa, P. O., Buerki, C., Lombardi, C., Imhof, R., and Hottiger, M. O. (2003) Transcriptional coactivation of nuclear factor-κB-dependent gene expression by p300 is regulated by poly(ADP)-ribose polymerase-1. *J. Biol. Chem.* **278,** 45,145–45,153
22. Fahrer, J., Kranaster, R., Altmeyer, M., Marx, A., and Burkle, A. (2007) Quantitative analysis of the binding affinity of poly(ADP-ribose) to specific binding proteins as a function of chain length. *Nucleic Acids Res.* **35,** e143
23. Muller, S., Berger, M., Lehembre, F., Seeler, J. S., Haupt, Y., and Dejean, A. (2000) c-Jun and p53 activity is modulated by SUMO-1 modification. *J. Biol. Chem.* **275,** 13,321–13,329
24. Haenni, S. S., Hassa, P. O., Altmeyer, M., Fey, M., Imhof, R., and Hottiger, M. O. (2008) Identification of lysines 36 and 37 of PARP-2 as targets for acetylation and auto-ADP-ribosylation. *Int. J. Biochem. Cell Biol.* **40,** 2274–2283
25. El-Andaloussi, N., Valovka, T., Toueille, M., Steinacher, R., Focke, F., Gehrig, P., Covic, M., Hassa, P. O., Schar, P., Hubscher, U., and Hottiger, M. O. (2006) Arginine methylation regulates DNA polymerase beta. *Mol. Cell* **22,** 51–62
26. Altmeyer, M., Messner, S., Hassa, P. O., Fey, M., and Hottiger, M. O. (2009) Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites. *Nucleic Acids Res.* **37,** 3723–3738
27. Kerscher, O. (2007) SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO Rep.* **8,** 550–555
28. Baba, D., Maita, N., Jee, J. G., Uchimura, Y., Saitoh, H., Sugasawa, K., Hanaoka, F., Tochio, H., Hiroaki, H., and Shirakawa, M. (2005) Crystal structure of thymine DNA glycosylase conjugated to SUMO-1. *Nature* **435,** 979–982
29. Takahashi, H., Hatakeyama, S., Saitoh, H., and Nakayama, K. I. (2005) Noncovalent SUMO-1 binding activity of thymine DNA glycosylase (TDG) is required for its SUMO-1 modification and colocalization with the promyelocytic leukemia protein. *J. Biol. Chem.* **280,** 5611–5621
30. Shen, T. H., Lin, H. K., Scaglioni, P. P., Yung, T. M., and Pandolfi, P. P. (2006) The mechanisms of PML-nuclear body formation. *Mol. Cell* **24,** 331–339
31. Hunter, T. (2007) The age of crosstalk: phosphorylation, ubiquitination, and beyond. *Mol. Cell* **28,** 730–738
32. Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A., and Sistonen, L. (2006) PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 45–50
33. Hietakangas, V., Ahlskog, J. K., Jakobsson, A. M., Hellesuo, M., Sahlberg, N. M., Holmberg, C. I., Mikhailov, A., Palvimo, J. J., Pirkkala, L., and Sistonen, L. (2003) Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Mol. Cell. Biol.* **23,** 2953–2968
34. Gregoire, S., Tremblay, A. M., Xiao, L., Yang, Q., Ma, K., Nie, J., Mao, Z., Wu, Z., Giguere, V., and Yang, X. J. (2006) Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. *J. Biol. Chem.* **281,** 4423–4433
35. Tremblay, A. M., Wilson, B. J., Yang, X. J., and Giguere, V. (2008) Phosphorylation-dependent sumoylation regulates estrogen-related receptor-alpha and -gamma transcriptional activity through a synergy control motif. *Mol. Endocrinol.* **22,** 570–584
36. Desterro, J. M., Rodriguez, M. S., and Hay, R. T. (1998) SUMO-1 modification of IκBα inhibits NF-κB activation. *Mol. Cell* **2,** 233–239
37. Cheng, J., Kang, X., Zhang, S., and Yeh, E. T. (2007) SUMO-specific protease 1 is essential for stabilization of HIF1α during hypoxia. *Cell* **131,** 584–595
38. Gregoire, S., and Yang, X. J. (2005) Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. *Mol. Cell. Biol.* **25,** 2273–2287
39. Stankovic-Valentin, N., Deltour, S., Seeler, J., Pinte, S., Vergoten, G., Guerardel, C., Dejean, A., and Leprince, D. (2007) An acetylation/deacetylation-SUMOylation switch through a phylogenetically conserved psiKXEP motif in the tumor suppressor HIC1 regulates transcriptional repression activity. *Mol. Cell. Biol.* **27,** 2661–2675
40. Shalizi, A., Gaudilliere, B., Yuan, Z., Stegmuller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J. W., and Bonni, A.

(2006) A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. *Science* **311,** 1012–1017
41. Hassa, P. O., and Hottiger, M. O. (2002) The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-κB in inflammatory disorders. *Cell. Mol. Life Sci.* **59,** 1534–1553
42. Hassa, P. O., Covic, M., Hasan, S., Imhof, R., and Hottiger, M. O. (2001) The enzymatic and DNA binding activity of PARP-1 are not required for NF-κB coactivator function. *J. Biol. Chem.* **276,** 45,588–45,597
43. Yang, S. H., and Sharrocks, A. D. (2004) SUMO promotes HDAC-mediated transcriptional repression. *Mol. Cell* **13,** 611–617
44. Stielow, B., Sapetschnig, A., Kruger, I., Kunert, N., Brehm, A., Boutros, M., and Suske, G. (2008) Identification of SUMO-dependent chromatin-associated transcriptional repression components by a genome-wide RNAi screen. *Mol. Cell* **29,** 742–754
45. Ouyang, J., Shi, Y., Valin, A., Xuan, Y., and Gill, G. (2009) Direct binding of CoREST1 to SUMO-2/3 contributes to gene-specific repression by the LSD1/CoREST1/HDAC complex. *Mol. Cell* **34,** 145–154
46. Kim, J. H., and Baek, S. H. (2009) Emerging roles of desumoylating enzymes. *Biochim. Biophys. Acta* **1792,** 155–162
47. Zhu, S., Goeres, J., Sixt, K. M., Bekes, M., Zhang, X. D., Salvesen, G. S., and Matunis, M. J. (2009) Protection from isopeptidase-mediated deconjugation regulates paralog-selective sumoylation of RanGAP1. *Mol. Cell* **33,** 570–580
48. Buerki, C., Rothgiesser, K. M., Valovka, T., Owen, H. R., Rehrauer, H., Fey, M., Lane, W. S., and Hottiger, M. O. (2008) Functional relevance of novel p300-mediated lysine 314 and 315 acetylation of RelA/p65. *Nucleic Acids Res.* **36,** 1665–1680
49. Liu, B., and Shuai, K. (2008) Regulation of the sumoylation system in gene expression. *Curr. Opin. Cell Biol.* **20,** 288–293
50. Comerford, K. M., Leonard, M. O., Karhausen, J., Carey, R., Colgan, S. P., and Taylor, C. T. (2003) Small ubiquitin-related modifier-1 modification mediates resolution of CREB-dependent responses to hypoxia. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 986–991
51. Carbia-Nagashima, A., Gerez, J., Perez-Castro, C., Paez-Pereda, M., Silberstein, S., Stalla, G. K., Holsboer, F., and Arzt, E. (2007) RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1α during hypoxia. *Cell* **131,** 309–323
52. Kaluz, S., Kaluzova, M., Liao, S. Y., Lerman, M., and Stanbridge, E. J. (2009) Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: A one transcription factor (HIF-1) show? *Biochim. Biophys. Acta* **1795,** 162–172
53. Peinado, H., Moreno-Bueno, G., Hardisson, D., Perez-Gomez, E., Santos, V., Mendiola, M., de Diego, J. I., Nistal, M., Quintanilla, M., Portillo, F., and Cano, A. (2008) Lysyl oxidase-like 2 as a new poor prognosis marker of squamous cell carcinomas. *Cancer Res.* **68,** 4541–4550

Received for publication May 18, 2009.
Accepted for publication July 2, 2009.

Supplementary Methods:

DNA binding assay

For *in vitro* DNA binding assays a biotinylated oligonucleotide (5′-GCTGTGGACCCTGCTGTGGGCTGGAGAACAAGGTGATCTGCG-3′) was annealed with a fully complementary oligonucleotide 5′-CGCAGATCACCTCCAGCCCACAGCAGGGTCCACAGC-3′ (control a). To mimic nicked or gapped DNA the biotinylated oligonucleotide was annealed with the oligonucleotide 5′-CGCAGATCACCTTGTTCTCCA-3′ and either 5′-GCGCACAGCAGGGTCCACAGC-3′ (control b, nicked fragment) or 5′-CGCAGATCACCTTGTTCT-P (gapped fragment). Biotinylated oligonucleotides were captured on Streptavidin Agarose beads (Novagen).

Immunofluorescence

For detection of proteins by immunofluorescence, HEK293T cells were fixed with freshly prepared Methanol:Acetic acid (3:1) mix for 5 min on ice, and washed with PBS. Unspecific binding sites were blocked with 0.05% Tween20 and 5% non-fat dry milk in PBS for 30 min at room temperature prior to staining with primary and FITC-conjugated secondary antibodies in the presence of blocking buffer. Pictures were taken with standard fluorescence microscope (Olympus Mx51, 40x, NA 1.3).

Reporter gene Assay

Mouse $PARP1^{+/+}$ or $PARP1^{-/-}$ mouse lung fibroblasts (MLF) cells were isolated from 129S/EV-PARP1+/+ and 129S/EV-PARP1−/− mice (Wang, Morrison, 1997). Only cell passages 2 to 10 were used for experiments. MLF were grown in DMEM Glutamax-I (Invitrogen) containing 4.5 g/L glucose, supplemented with 10% FCS (Invitrogen), 50 units/mL penicillin, 50 µg/mL streptomycin (Sigma), and α-naphthylacetic acid (Invitrogen). Cells were grown in 5% CO2 at 37°C in a humidified incubator. MLF were transfected with polyethylenimine as previously described. Because of differences in transfection efficiencies, an expression plasmid of CMV driven Renilla luceriferase was cotransfected as a transfection efficiency control, and luciferase activities were normalized based on Renilla light units. Luciferase activity was measured with the pSG5C-CA9 reporter gene (described in Kopacek J, et al. (2005). Biochim Biophys Acta 1729: 41-49).

Plasmids

Human HIF1-α (aa 365-805) was amplified from pENTR-d hHIF1-α (aa 5-805) and cloned into a pGEX6P3 vector with BamHI and XhoI restriction enzymes.

Protein purification

GST-hHIF1-α (aa 365-805) was expressed in bacteria and purified according to standard protocols using glutathione sepharose 4B (GE healthcare).

Immunoprecipitation

Sumoylated PARP1 was purified as described in the material and methods section. Either sumoylated HA-PARP1 or desumoylated HA-PARP1 was incubated on protein G sepharose beads (GE healthcare) together with anti-HA antibody. After 1.5 h incubation time, the beads were washed and 5 ug of purified GST-HIF1-α (aa 365-805) was added for 2h. The beads were washed with IP-buffer (50 mM Tris pH 8.0, 170 mM NaCl, 0.5% NP-40, Protease inhibitors) and subjected to SDS-PAGE, followed by western blot analysis.

Supplementary Figures:

Supplementary Figure 1:

(A) HA-PARP1-Ubc9 fusion protein was ectopically expressed with either myc-SUMO1 or myc-SUMO3 in HEK293T. Cell extracts were examined with anti-HA and anti-myc antibody. (B) HA-PARP1-Ubc9 or HA-PARP1-Ubc9 C93S were transfected along with myc-SUMO3 into HEK293T cells. Whole cell extracts were taken and analyzed with anti-HA antibody (C) Immunoprecipitation of ectopically expressed HA-PARP1-Ubc9 fusion protein, expressed in HEK293T with or without myc-SUMO3. Cell extracts were examined with anti-HA and anti-myc antibody.

Supplementary Figure 2:

(A) GST-pulldown with GST alone, conjugation deficient GST-SUMO-AA, or GST-p65 against whole cell extracts from HEK293T. Detection of PARP1-binding was done with PARP1 western blot. GST was used as negative control, GST-p65 as positive control for PARP1 interaction. A coomassie stain of the western blot membrane is shown. (B) GST-pulldown as in (A), but with purified PARP1. (C) GST-pulldown with more stringent conditions than in (B), but with recombinant purified TDG as positive control for non-covalent interaction.

Supplementary Figure 3:

(A) An *in vitro* sumoylation assay with purified PARP1 was carried out without or with EcoRI-linker DNA in the reaction. (B) Three different biotin-tagged oligonucleotides were bound to *in vitro* translated ^{35}S-Methionine labeled and sumoylated PARP1. Precipitation of the oligonucleotides was performed with Streptavidin-Agarose. Bound proteins were separated through SDS-PAGE and detected with autoradiography.

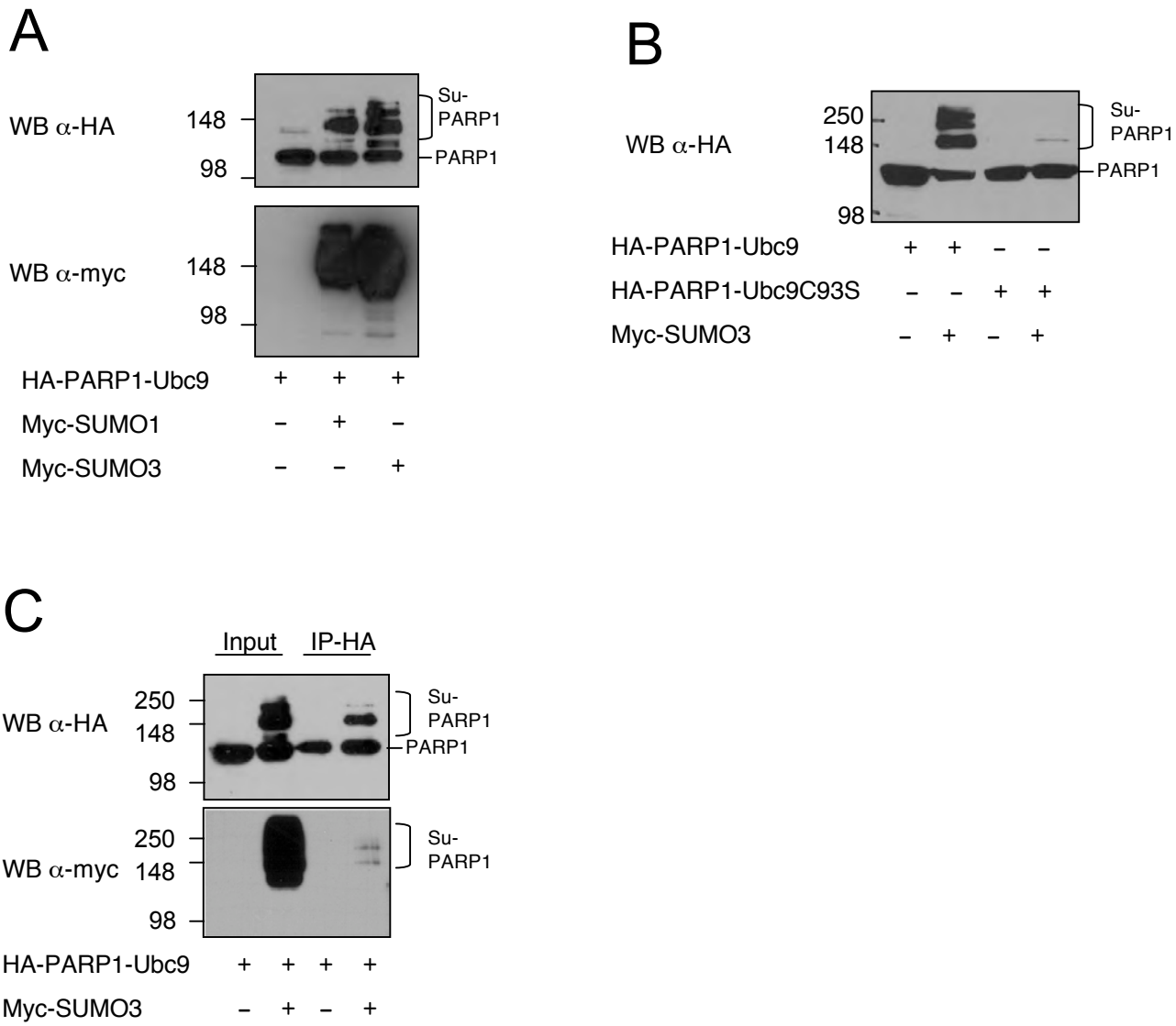
Supplementary Figure 4:

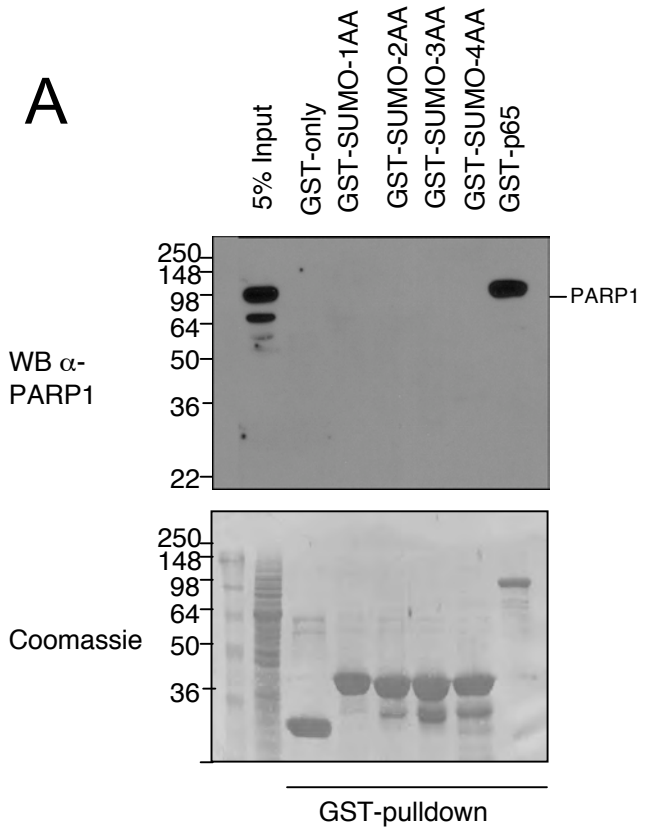
(A) Immunofluorescence staining of untransfected or with myc-SUMO3 transfected HEK293T cells. The cells were fixed 28 h after transfection and stained with the anti-SUMO2/3 (18H8) or anti-PAR (10H) antibody. (B) Immunofluorescence staining of untransfected or with myc-SUMO3 transfected HEK293T cells. The cells were treated with 0.5 mM H_2O_2 for 10 min, fixed and stained with the anti-SUMO2/3 (18H8) or anti-PAR (10H) antibody. (C) Immunofluorescence staining of untransfected or with myc-SUMO3

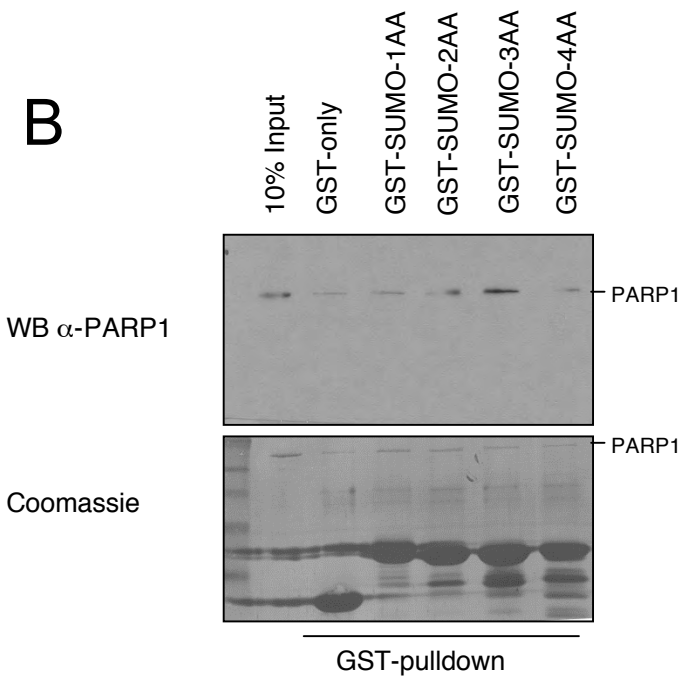
transfected HEK293T cells. Cells were fixed 28 h after transfection and stained with anit-PARP1 (H250) or anti-myc (9E10) antibody.

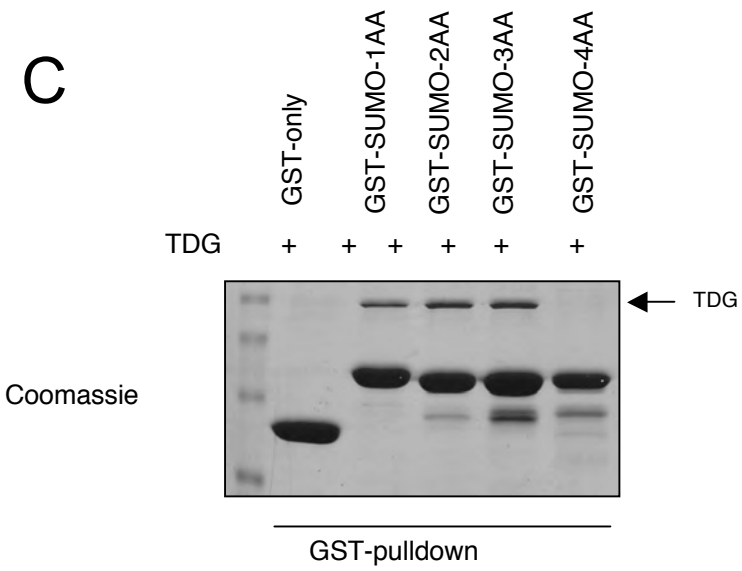
Supplementary Figure 5:

(A) *In vitro* acetylation assay with PARP1 wild-type or with acetylation-deficient PARP1 KQR (K498R, K505R, K521R, K524R) mutant. Western blot was done with monoclonal E4 antibody and anti-PARP1 antibody. The antibody was generated against an acetylated peptide corresponding to residues K498, K505 and K508 in human PARP1 sequence. Control of equal inputs of PARP1 was done through coomassie stain of the western blot membrane. (B) CAIX reporter plasmid is induced with SUMOylation deficient PARP1. Primary mouse lung fibroblasts (MLF) were transfected with HA-PARP1 wt or HA-PARP1 K486R along with CAIX reporter plasmids and Renilla-Luciferase for normalization. Cells were induced with hypoxia mimicking drug Ciclopirox-olamine for 8h. (C) SUMO2/3 conjugation is induced after hypoxia. K562 whole cell extracts were examined with SUMO 2/3 antibody after hypoxic induction for 28h at 0.2% O_2. (D) HEK293T cells were transfected with HA-PARP1 and myc-SUMO3 expression plasmids. 20 hours after transfection, cells were exposed to hypoxia (0.2%O_2) for 28 h. Whole cell extracts were examined by western blot using anti-myc and anti-PARP1 antibodies. (E) HA-PARP1 was sumoylated (Su-PARP1) or desumoylated (deSu-PARP1) *in vitro* as described in Fig. 3A, and subsequently immunoprecipitated using anti-HA antibody. As control, only beads with the anti-HA antibody was used (no protein). After extensive washing, beads were incubated with purified GST-HIF1-α (aa 365-805). Co-immunoprecipitated GST-HIF1-α was detected by western blot using an anti-GST antibody.

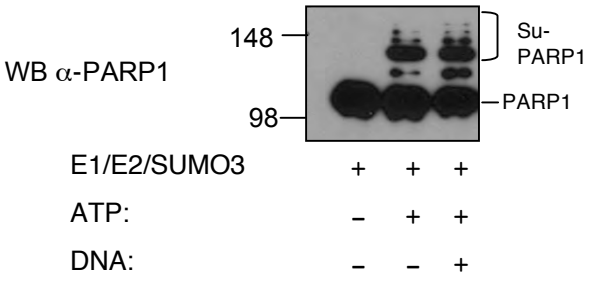

A
WB α-HA
148
98
Su-
PARP1
PARP1
WB α-myc
148
98
HA-PARP1-Ubc9 + + +
Myc-SUMO1 – + –
Myc-SUMO3 – – +
B
WB α-HA
250
148
98
Su-
PARP1
PARP1
HA-PARP1-Ubc9 + + – –
HA-PARP1-Ubc9C93S – – + +
Myc-SUMO3 – + – +
C
Input
IP-HA
WB α-HA
250
148
98
Su-
PARP1
PARP1
WB α-myc
250
148
98
Su-
PARP1
HA-PARP1-Ubc9 + + + +
Myc-SUMO3 – + – +


A
5% Input
GST-only
GST-SUMO-1AA
GST-SUMO-2AA
GST-SUMO-3AA
GST-SUMO-4AA
GST-p65
250
148
98
64
50
36
22
PARP1
WB α-PARP1
Coomassie
GST-pulldown

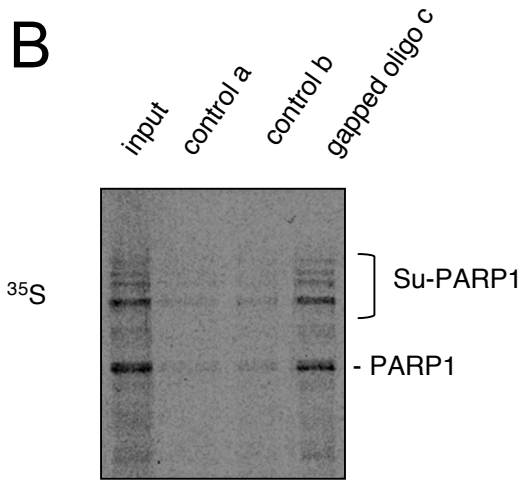

B
10% Input
GST-only
GST-SUMO-1AA
GST-SUMO-2AA
GST-SUMO-3AA
GST-SUMO-4AA
PARP1
WB α-PARP1
PARP1
Coomassie
GST-pulldown

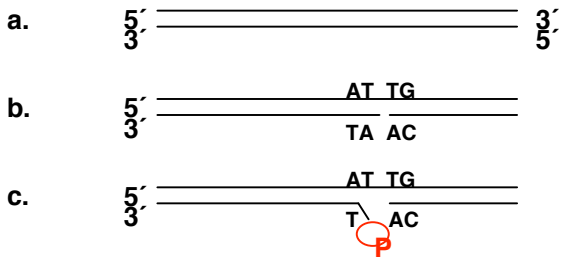

C
GST-only
GST-SUMO-1AA
GST-SUMO-2AA
GST-SUMO-3AA
GST-SUMO-4AA
TDG + + + + + +
TDG
Coomassie
GST-pulldown

A

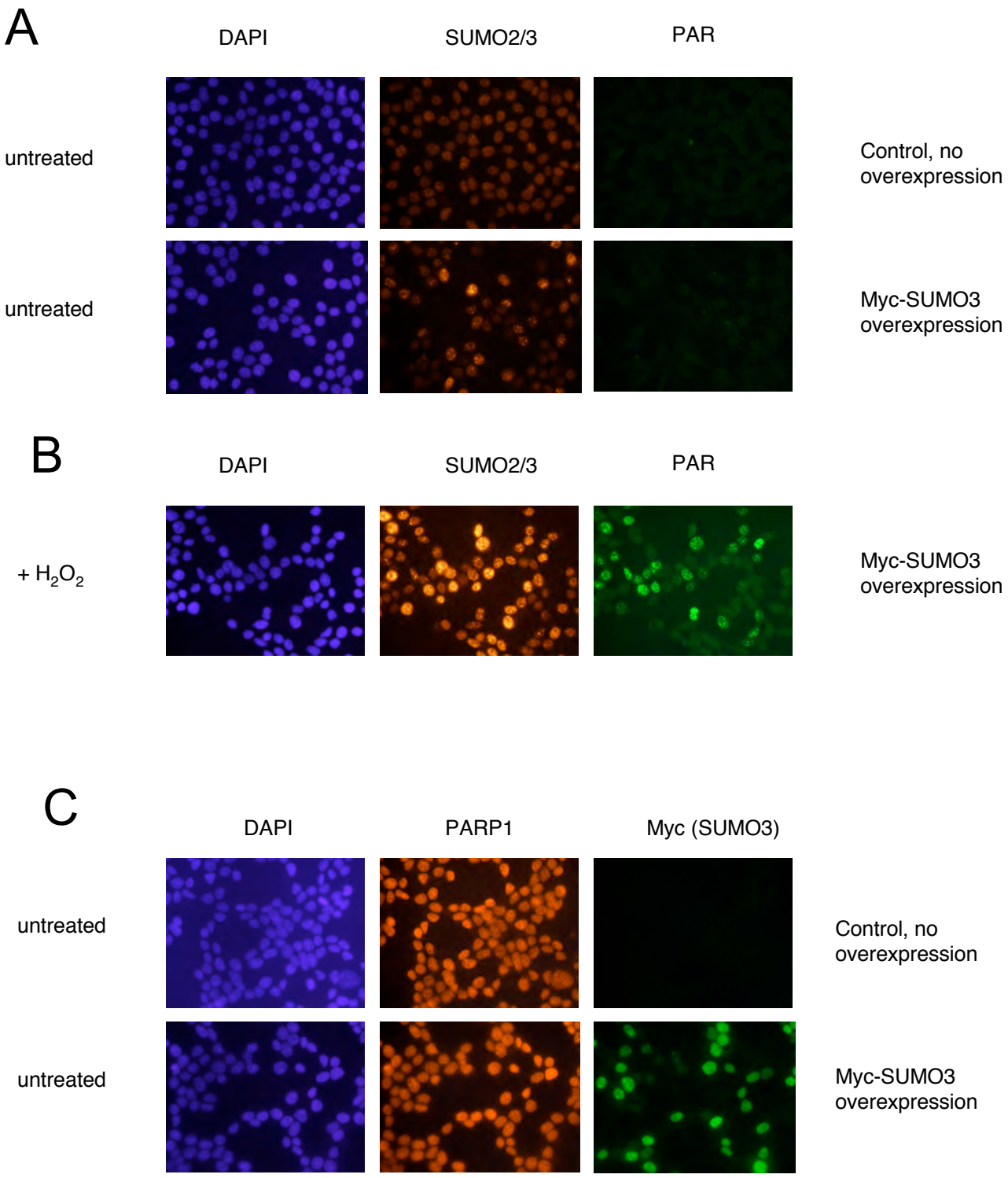

148
98
WB α-PARP1
Su-
PARP1
PARP1
E1/E2/SUMO3 + + +
ATP: – + +
DNA: – – +

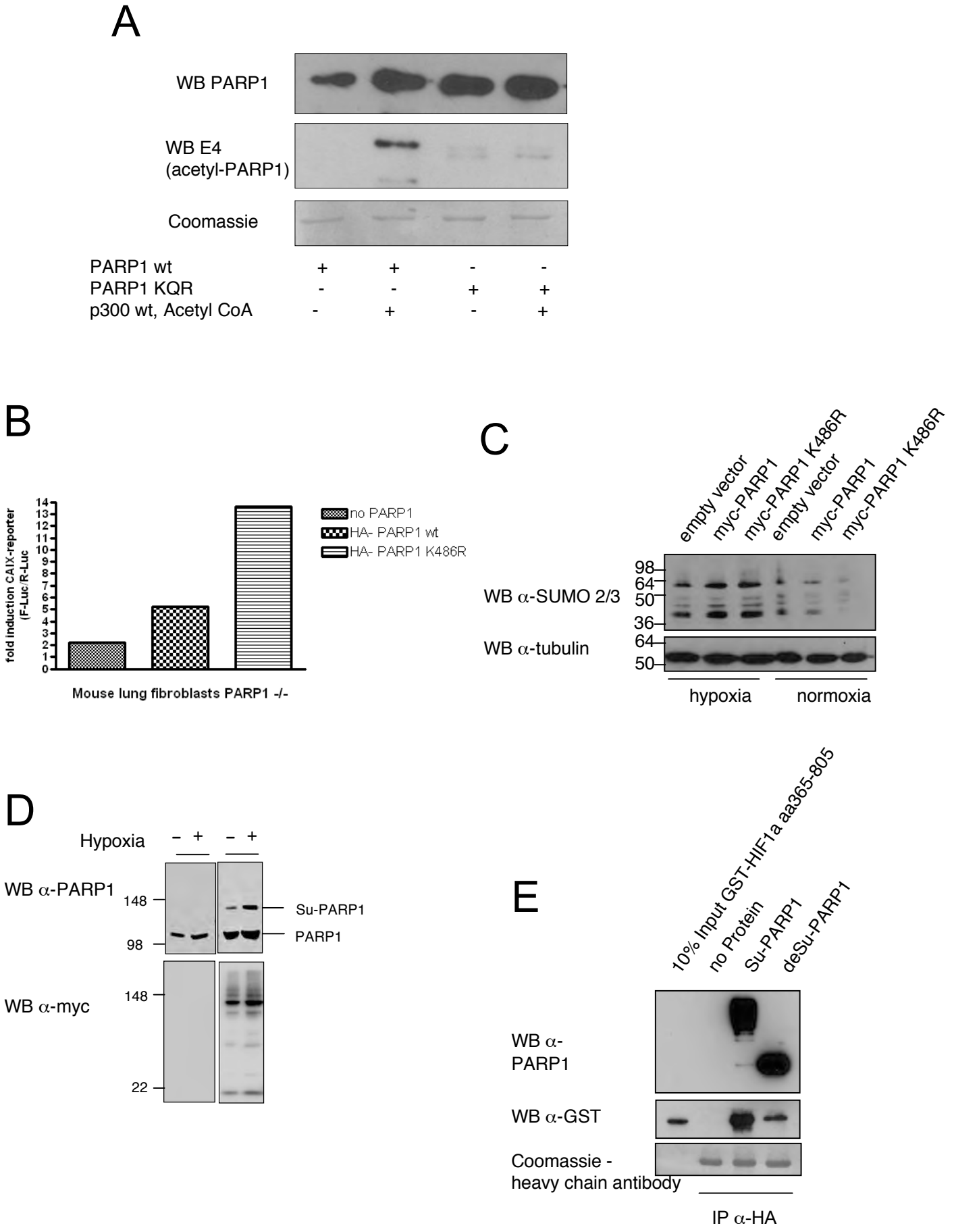
B


input
control a
control b
gapped oligo c
35S
Su-PARP1
- PARP1


a.
5´
3´
3´
5´
b.
AT TG
TA AC
c.
T
P

Supplementary Fig. 4




A
WB PARP1
WB E4
(acetyl-PARP1)
Coomassie
PARP1 wt + + - -
PARP1 KQR - - + +
p300 wt, Acetyl CoA - + - +
B
fold induction CAIX-reporter (F-Luc/R-Luc
no PARP1
HA- PARP1 wt
HA- PARP1 K486R
Mouse lung fibroblasts PARP1 -/-
C
empty vector
myc-PARP1
myc-PARP1 K486R
empty vector
myc-PARP1
myc-PARP1 K486R
WB α-SUMO 2/3
WB α-tubulin
98
64
50
36
64
50
hypoxia
normoxia
D
Hypoxia - + - +
WB α-PARP1
148
98
Su-PARP1
PARP1
WB α-myc
148
22
E
10% Input GST-HIF1a aa365-805
no Protein
Su-PARP1
deSu-PARP1
WB α-PARP1
WB α-GST
Coomassie - heavy chain antibody
IP α-HA

Nucleic Acids Research Advance Access published June 4, 2010

Nucleic Acids Research, 2010, 1–13
doi:10.1093/nar/gkq463

PARP1 ADP-ribosylates lysine residues of the core histone tails

Simon Messner[1,2], Matthias Altmeyer[1,2], Hongtao Zhao[3], Andrea Pozivil[1], Bernd Roschitzki[4], Peter Gehrig[4], Dorothea Rutishauser[4], Danzhi Huang[3], Amedeo Caflisch[3] and Michael O. Hottiger[1,*]

[1]Institute of Veterinary Biochemistry and Molecular Biology, [2]Life Science Zurich Graduate School, Molecular Life Science Program, [3]Department of Biochemistry and [4]Functional Genomics Center Zurich, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Received February 24, 2010; Revised April 27, 2010; Accepted May 10, 2010

ABSTRACT

The chromatin-associated enzyme PARP1 has previously been suggested to ADP-ribosylate histones, but the specific ADP-ribose acceptor sites have remained enigmatic. Here, we show that PARP1 covalently ADP-ribosylates the amino-terminal histone tails of all core histones. Using biochemical tools and novel electron transfer dissociation mass spectrometric protocols, we identify for the first time K13 of H2A, K30 of H2B, K27 and K37 of H3, as well as K16 of H4 as ADP-ribose acceptor sites. Multiple explicit water molecular dynamics simulations of the H4 tail peptide into the catalytic cleft of PARP1 indicate that two stable intermolecular salt bridges hold the peptide in an orientation that allows K16 ADP-ribosylation. Consistent with a functional cross-talk between ADP-ribosylation and other histone tail modifications, acetylation of H4K16 inhibits ADP-ribosylation by PARP1. Taken together, our computational and experimental results provide strong evidence that PARP1 modifies important regulatory lysines of the core histone tails.

INTRODUCTION

Histone proteins form the nucleosome, which is the fundamental repeating unit of chromatin (1,2). Each nucleosome contains two heterodimers of the core histones H2A and H2B, one tetramer of the core histones H3 and H4, and 146 bp of DNA (3). Dynamic chromatin structures are governed in part by post-translational modifications of the histones, modification of nucleotides, remodeling of nucleosomes, and by non-coding RNAs or non-histone DNA-binding proteins (4). The amino-terminal tails of the core histone proteins protrude from the nucleosome. They appear to be unstructured and are believed to participate in the formation of higher order chromatin structures by mediating internucleosomal interactions and to contact the linker DNA (3,5). A large number of residues within the histones are modified by post-translational modifications including acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation, which occur in distinct patterns (6). Recent work has provided compelling evidence that these modifications influence the functional properties of chromatin.

Histones have long been known as substrates for ADP-ribosylation *in vivo* (7). Histones isolated from rat liver nuclei and HeLa cells incubated with radioactive NAD^+ revealed that the linker histone H1 and all core histones, H2A, H2B, H3 and H4, are ADP-ribosylated, although to a variable extent (8–12). An unresolved issue regarding the mechanism of ADP-ribosylation of histones is whether this modification primarily occurs at the globular histone domains or at their unstructured amino-terminals tails. Moreover, unconfirmed ADP-ribose acceptor amino acids have previously only been proposed for H1 and H2B, but not for H2A, H3 and H4 (11,13–15).

Poly(ADP-ribose) polymerase 1 (ARTD1/PARP1) is a nuclear chromatin-associated multifunctional enzyme that is present in most eukaryotes apart from yeast (16). The enzyme is responsible for most of the cellular poly(ADP-ribose) formation. Targets of PARP1's enzymatic activity include a variety of nuclear proteins, most prominently PARP1 itself, as well as histone proteins (17). Among the six PARP family members, PARP2 has the highest similarity with PARP1 (43% sequence identity

*To whom correspondence should be addressed. Tel: +41 44 635 54 74; Fax: +41 44 635 68 40; Email: hottiger@vetbio.uzh.ch

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

in the catalytic domain) (18). PARP2 displays similar automodification properties as PARP1 and may account for the residual poly(ADP-ribose) synthesis observed in PARP1 knockout mice.

Recently, we showed that individual lysine residues of PARP1 and PARP2 function as acceptor sites for auto-ADP-ribosylation and not, as previously assumed, glutamic acid residues (19,20). Here, we report PARP1-mediated ADP-ribosylation of the core histone proteins. We found that PARP1, but not PARP2, ADP-ribosylates core histone proteins at their amino-terminal tails. Mass spectrometry of ADP-ribosylated histone peptides revealed that the lysine residues K13 of H2A, K30 of H2B, K27 and K37 of H3, as well as K16 of H4, are specifically ADP-ribosylated by PARP1. Acetylation of H4K16 or mutation of this residue to an alanine abrogated ADP-ribosylation. Molecular dynamics (MD) simulations of tetra- and octapeptide segments of the amino terminal tail of H4 indicated that two positively charged side chains at positions n and $n+1$ in the histone sequence, which point in opposite directions, are engaged in favorable electrostatic interactions with two acidic PARP1 residues at the positions 988 and 756, buried in the catalytic cleft and on a loop at the entrance of the cleft, respectively. Taken together, our results reveal that PARP1 specifically modifies lysine residues of the core histone tails, which are known to control chromatin structure and function.

MATERIALS AND METHODS

Chemicals and antibodies

^{32}P-NAD$^+$ was purchased from PerkinElmer. NAD$^+$ was obtained from Sigma-Aldrich. Anti-PAR (LP-96-10) antibody was from Becton Dickinson. Anti-PARP1 antibody was generated in this laboratory. PARP-inhibitor DAM-TIQ-A was obtained from Alexis Biochemicals, PJ34 was purchased from Enzo Life Science, 3-amino-benzamide (3AB) was from Sigma-Aldrich. Peptides were from PiProteomics. Probond^{TM} nickel beads were from Invitrogen. Glutathione-Sepharose 4B affinity beads were from GE-healthcare.

Plasmids

The baculovirus expression vectors pQE-TriSystem (Qiagen) and BacPak8 (Clontech) were used for the expression of recombinant proteins in Sf21 cells as described (21,22). pGEX-2T vectors (GE healthcare) were used for the cloning and expression of GST-fusion proteins. Full-length and truncated histone proteins were expressed in pET3a or pET3d vectors, as described (3). Sequencing of plasmids was performed by Microsynth (Balgach, CH).

Cloning, expression and purification of recombinant proteins

Human PARP1 and PARP2 were cloned, expressed and purified as described (19). Full-length histone proteins were generated as described (3). GST-histone tail fusion proteins were generated by PCR and cloned into pGEX-2T vector. Truncated and mutated versions of GST-fusion proteins were generated by cloning with BstBI and EcoRI restriction sites. Primers for PCR and direct cloning were obtained from Sigma-Aldrich and Microsynth. pGEX-2T plasmids were transformed into BL21(DE3) bacteria and expression was induced by addition of 1 mM IPTG into LB-medium for 3 h at 30°C. After centrifugation and resuspension in EBC-buffer (120 mM NaCl, 50 mM Tris–pH 8.0, 0.5% NP-40, 5 mM DTT, 1 mM PMSF), bacteria were lysed by the addition of lysozyme (0.5 mg/ml) and the DNA was sheared by a French Press. After centrifugation the supernatant was taken and the proteins were bound to GST-beads and washed extensively with EBC buffer. The GST-beads with the bound GST-fusion proteins were equilibrated with ADP-ribosylation buffer (50 mM Tris–HCl, pH 8.0, 4 mM $MgCl_2$, 250 μM DTT, 20 mM NaCl, 1 μg/ml protease inhibitors pepstatin, leupeptin and bestatin) and after extensive washing eluted from the GST-beads with 10 mM reduced glutathione in ADP-ribosylation buffer.

ADP-ribosylation assays

^{32}P-NAD$^+$ ADP-ribosylation was performed as previously described (19). Briefly, 20 μg histone mix from calf thymus (Roche) were incubated with 10 pmol PARP1 or PARP2 in a 25 μl reaction, containing 5 pmol annealed EcoRI-linker DNA and 100 nM ^{32}P-NAD$^+$ in ADP-ribosylation buffer (50 mM Tris–HCl, pH 8.0, 4 mM $MgCl_2$, 250 μM DTT, 20 mM NaCl, 1 μg/ml protease inhibitors pepstatin, leupeptin and bestatin) for 10 min at 30°C. ADP-ribosylation of full-length or truncated single histones was performed with 3 μg full-length or truncated histone proteins and 10 pmol PARP1 in a 25 μl reaction. ADP-ribosylation of GST-histone tail fusion proteins was performed with 1.5 μg purified fusion protein, together with 10 pmol of PARP1. An amount of 5 pmol EcoRI-linker DNA was always included into the reaction to activate PARP1 enzymatic activity. ADP-ribosylation assays with full-length or truncated histones were resolved by a 10–20% acrylamide gradient SDS-gel of 15 cm length (Amersham). Assays with GST-fusion proteins were resolved on standard 12% acrylamide mini-gels (Hoefer). Radiolabeled proteins were visualized by exposure to X-ray films or by quantification through the phosphoimager system (Molecular Dynamics).

For mass spectrometry, 22 nmol biotinylated H2A (aa 3–23), H2B (aa 18–37), H3 (aa 23–42), H4 (aa 1–22) or H4K16ac (aa 1–22) peptides were incubated with 10 pmol PARP1, 5 pmol annealed EcoRI-linker and 100 or 500 μM NAD$^+$ for 15 min at 30°C in ADP-ribosylation buffer without protease inhibitors. The reaction was stopped by the addition of 3AB to a final concentration of 20 mM and subsequently 1 μg GST-ARH3 was added to the reaction and further incubated for 1 h at 30°C. The samples were acetone precipitated and the pellet was dissolved in distilled water.

GST-pulldown

Glutathione–sepharose affinity beads were incubated with a bacterial extract of the GST-fusion protein expression in EBC-buffer. After extensive washing, the GST-beads were equilibrated with ADP-ribosylation buffer containing 50 mM NaCl. After washing, 10 pmol purified PARP1 was added to the beads in a total volume of 300 µl ADP-ribosylation buffer. The protein mixture was incubated for 2 h at 4°C on rolls to allow for protein–protein interaction. The samples were washed again with ADP-ribosylation buffer and resolved on standard SDS–PAGE and subsequent western blotting with anti-PARP1 antibody.

Microvolume C-18 reversed phase purification

An amount of 22 nmol H4-peptide (aa 1–22) was incubated with 10 pmol PARP1, 5 pmol EcoRI-linker DNA and 100 nM ^{32}P-NAD$^+$ in 25 µl ADP-ribosylation buffer for 15 min at 30°C. The reaction was stopped by the addition of 3AB to a final concentration of 20 mM. The C18 reversed phase ZipTip (Millipore) was pre-wetted with 100% methanol and equilibrated with 3% (v/v) acetonitrile. The peptides were bound onto the column and subsequently washed extensively with 3% (v/v) acetonitrile. Bound peptides were eluted by 60% (v/v) acetonitrile directly into scintillation liquid. In the control reaction, peptides were added after the addition of 3AB to the reaction. Scintillation counts were measured and normalized to the control reaction and the relative increase in scintillation counts was calculated (cpm).

Mass spectrometry

Electron transfer dissociation (ETD) experiments were performed on a hybrid LTQ Orbitrap XL ETD mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an Eksigent nano LC system (Eksigent Technologies) and the samples were analyzed by reversed-phase liquid chromatography nanospray tandem mass spectrometry (LC–MS/MS).

Peptides were resuspended in 3% ACN and 0.2% formic acid, loaded from a cooled (10°C) Spark Holland autosampler (Emmen, Holland) and separated using an ACN/water solvent system containing 0.2% formic acid with a flow rate of 200 nl/min. Separation of the peptides was performed on a 10-cm long fused silica column (75 µm i.d.; BGB Analytik) in-house packed with 3 µm, 200 Å pore size C18 resin (Michrom BioResources, CA). Elution was achieved using a gradient of 3–48% ACN in 35 min, 48–80% ACN in 4 min and 80% ACN for 7 min.

One scan cycle was comprised of a survey full scan MS spectra from m/z 300 to 2000 acquired in the FT-Orbitrap with a resolution of $R = 60\,000$ at m/z 400, followed by up to four sequential data-dependent ETD MS/MS scans with detection of the ETD fragment ions in the linear ion trap. AGC target values were 5×10^5 for full FTMS scans, 10^4 for ion trap MSn scans. Anion target value was 10^6 and supplementary activation was employed to enhance the fragmentation efficiency for doubly-charged precursors and charge state dependent ETD time was enabled. Data dependent decision tree was used in order to control ETD dissociation based on charge state and m/z. The ETD reaction time was set at 100 ms and the isolation width was m/z 2. For all experiments dynamic exclusion was used with one repeat count, 30-s repeat duration, and 10-s exclusion duration.

The instrument was calibrated externally according to the manufacturer's instructions. Orbitrap mass spectra were acquired using internal lock mass calibration on m/z 429.088735 and 445.120025. Spectra generated by ETD were processed using Mascot Distiller 2.2 (Matrix Science) and data was searched against a SwissProt human database using Mascot 2.2.0 to find best matching sequences. Detailed spectra analysis was done by manual evaluation.

Molecular dynamics

It is computationally prohibitive to dock the full-length histone tails to PARP1. Therefore, only the relevant tetra- and octapeptide segments of the histone tails (abbreviated H-peptides hereafter) were taken into account in the present study. A two-step procedure was used to investigate the binding of the H-peptides to PARP1 (PDB ID: 1A26). Preliminary binding modes of the flexible H-peptides into the rigid structure of the catalytic domain of PARP1 were obtained by an in-house developed docking program, which uses a combination of simulated annealing and genetic algorithm optimization of position, orientation, and rotatable bonds of the ligand (Zhao *et al.,* unpublished). Explicit solvent MD simulations of the H-peptide/PARP1 complexes were then used to investigate the structural stability of the poses obtained by docking. In both docking and MD simulations, the N-terminus and C-terminus of the H-peptides were capped by neutral groups (acetyl and *N*-methlyamide, respectively) to take into account the fact that they belong to a longer polypeptide chain. To reproduce physiological pH conditions, the side chains of aspartates and glutamates were negatively charged, those of lysines and arginines were positively charged, while all other residues were considered neutral. The MD simulations were performed with the program NAMD (23) using the all-atom CHARMM PARAM27 force field (24) and the TIP3P model of water (25). The H-peptide/PARP1 complexes were inserted into a cubic water box, with a minimal distance of 12 Å between any solute atom and the boundary of the box. Chloride and sodium ions were added to neutralize the system and approximate a salt concentration of 150 mM. The water molecules overlapping with the solute atoms or the ions were removed, if the distance between the water oxygen and any atom of the complex or any ion was smaller than 2.4 Å. Periodic boundary conditions were applied to avoid finite-size effects. Electrostatic interactions were calculated within a cutoff of 12 Å, while long-range electrostatic effects were taken into account by the particle mesh Ewald summation method (26). Van der Waals interactions were treated with the use of a switch function starting at 10 Å and turning off at 12 Å,

which is the default of the all-atom CHARMM force field. The temperature was kept constant at 300 K by the Langevin temperature control with a damping coefficient of 5 ps^{-1}, while the pressure was held constant at 1 atm by applying a pressure piston. Before the production runs, water molecules and ions were subjected to energy minimization for 6000 steps, and a 1-ns equilibration with harmonic constraints applied to the positions of the C-alpha atoms. The covalent bonds involving hydrogen atoms were constrained by means of the SHAKE algorithm, and the dynamics were integrated with a time step of 2 fs. Snapshots were saved every 2 ps for trajectory analysis. Two MD runs with different initial distribution of velocities were carried out for each of the H4 peptides AKRH and AKRHRKIL for a total simulation time of 150 ns for each peptide. Analysis of the trajectories was carried out with the programs CHARMM (27) and WORDOM (28).

RESULTS

PARP1 modifies core histones

Recently, we reported the ADP-ribosylation of distinct lysine residues of PARP1 and PARP2 *in cis* (19,20). Since core histones were described earlier to be modified by PARP1 *in vitro* and *in vivo* [(16) and Supplementary Figure S1A and B], we set out to investigate which residues of H2A, H2B, H3 and H4 would be modified by PARP1, and possibly also by PARP2 *in trans*. First, human PARP1 and PARP2 were expressed and purified from insect cells using the baculovirus-system and were subsequently incubated with full-length histones isolated from calf thymus together with 100 nM radiolabeled NAD^+. Although automodification of both PARP1 and PARP2 was easily detectable, only PARP1 displayed detectable *trans*-ADP-ribosylation of all four core histones, indicating a clear difference in the substrate specificity between PARP1 and PARP2 (Figure 1A). Similar experiments with bacterially expressed and purified single histone proteins revealed that, indeed, all four histones are modified by PARP1 (Figure 1B).

PARP1 mono- and poly(ADP-ribosyl)ates amino-terminal tails of core histone proteins covalently

Earlier reports suggested that histones are mainly ADP-ribosylated at their amino-terminal tails (13,29). To test whether PARP1-mediated ADP-ribosylation occurred at the amino-terminal tails or at the globular histone folds, we incubated the globular domains of histones H2B, H3 and H4 together with PARP1 and compared their ADP-ribosylation to the full-length counterpart (Supplementary Figure S2A). ADP-ribosylation of the globular domains was reduced in comparison to full-length histones, implying that indeed the amino-terminal tails are required for modification by PARP1. We then expressed the different histone tails as GST fusion proteins in bacteria and incubated them with purified PARP1 and radiolabeled NAD^+. PARP1 was able to modify all four histone tails; whereas comparable PARP2-mediated ADP-ribosylation of histone tails was not detectable (Figure 1C and Supplementary Figure S2B). This finding suggests that the core histone tails are substrates specifically for PARP1 but not for PARP2 *in vitro*. Previous reports indicated that histones can interact with poly(ADP-ribose) non-covalently via a PAR-binding motif (30). Since the tested histone tails we analyzed did not contain this motif, it is highly unlikely that the observed labeling represented non-covalently attached PAR. To exclude this notion experimentally, the histone tail fusion proteins were added to the ADP-ribosylation reaction either together with PARP1 or after the enzymatic reaction had been stopped by addition of the PARP-inhibitor 3AB. The addition of the histone tails after the reaction did not result in their modification (Figure 1D, right panel), implying that the observed ADP-ribosylation is a covalent modification. To test whether the observed modification is an enzymatic reaction, the reaction was repeated in presence of the PARP-inhibitor PJ34. Increasing amounts of PJ34 abolished *trans*-ADP-ribosylation of the H2B tail, as well as automodification of PARP1 (Figure 1E). Similar results were obtained with a second PARP-inhibitor, DAM-TIQ-A (Figure 1E). Taken together these results indicate that PARP1 catalyzes the covalent ADP-ribosylation of histone tails.

PARP1 is a mono- and a poly(ADP-ribosyl) transferase (16). To determine whether the histone tails can be mono and/or poly(ADP-ribosyl)ated by PARP1, we incubated the histone tails with PARP1 in the presence of increasing amounts of NAD^+. Poly(ADP-ribosyl)ation was determined by immunoblot analysis using an anti-PAR antibody, which recognizes only polymers of ADP-ribose (PAR). Both automodification of PARP1 and poly(ADP-ribosyl)ation of the histones were detected, indicating that PARP1 can attach long polymers of ADP-ribose onto histone tails (Figure 1F). Notably, the length of poly(ADP-ribose) chains of the histones increased proportionally to the amounts of NAD^+, which led to retarded migration of the modified proteins due to an increased molecular weight (Figure 1F, filled asterisk).

ADP-ribosylation was earlier described to occur on glutamates of H2B and H1 (11,15). These findings were never directly attributed to PARP1 nor were they experimentally confirmed by mass spectrometry or amino-acid substitutions. The PARP1-mediated ADP-ribosylation we describe here occurred on the basic amino-terminal histone tails, most of which do not contain glutamates (Figure 2A). One exception is the tail of histone H2B that contains one single glutamate. Mutation of this residue to alanine did not affect the levels of incorporated ADP-ribose onto the H2B tail by PARP1 (Supplementary Figure S2C), suggesting that glutamates are dispensable and that additional residues can be efficiently ADP-ribosylated by PARP1 *in trans*. Furthermore, when we incubated poly-L-lysine or poly-L-glutamate with purified PARP1 and measured incorporated ADP-ribose, lysines but not glutamates were modified by PARP1 (Supplementary Figure S2D). These findings are consistent with our previous reports (19,20) and provided additional evidence that lysines are likely the primary target sites for PARP1-mediated ADP-ribosylation.

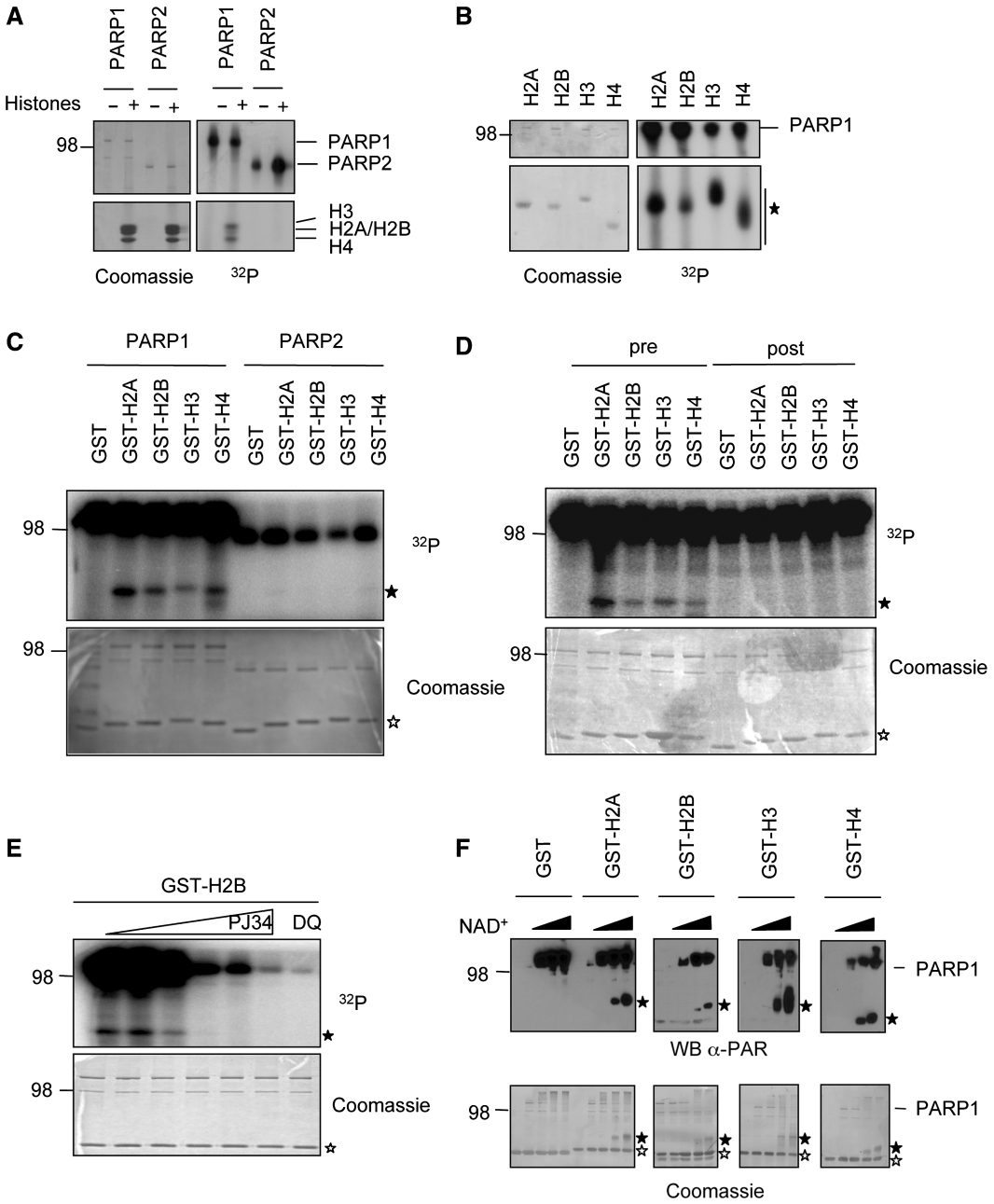


Figure 1. PARP1 covalently modifies all four core histone tails. (**A**) PARP1 *trans*-ADP-ribosylates histones isolated from calf thymus. An amount of 10 pmol recombinant PARP1 or PARP2 were incubated for 15 min (PARP1) or 30 min (PARP2) at 30°C with 5 pmol EcoRI-linker DNA, 100 nM radiolabeled NAD^+ and 20 µg extracted histones from calf thymus. The reaction was stopped by addition of SDS-lysis buffer and the proteins were resolved on a 10–20% SDS–PAGE gradient gel. The gel was stained with Coomassie-R250 and incorporated ^{32}P was visualized by autoradiography. (**B**) Recombinant expressed and purified histones (3 µg) were ADP-ribosylated by PARP1 as in Figure 1A. (**C**) An amount of 1.5 µg of each purified GST-histone tail were used in PARP1 or PARP2 mediated *trans*-ADP-ribosylation reactions for 5 min at 30°C. (**D**) An amount of 1.5 µg of each purified GST-histone tail were used in PARP1 mediated *trans*-ADP-ribosylation reactions for 5 min at 30°C. Histone tails were either included during the reaction (pre) or added after the reaction had been stopped with a 100-fold excess of 3AB over radiolabeled NAD^+ (post) to exclude non-covalent interaction of the histone tails with PAR. (**E**) *Trans*-ADP-ribosylation of the H2B tail is inhibited by the PARP-inhibitors PJ34 (0.01–100 µM) and DAM-TIQ-A (10 µM). (**F**) GST-histones were incubated with PARP1, EcoRI linker and increasing concentrations of NAD^+ (0, 10, 100, 400 µM) for 10 min at 30°C. Poly(ADP-ribose) formation was assessed through western blotting with anti-PAR (LP-96-10) antibody. Unmodified GST-histone tails are marked by an empty asterisk, PARylated GST-histone tails are marked by a filled asterisk.

Identification of ADP-ribose acceptor sites within histone tails

As the amino-terminal histone tails are frequently targeted by a variety of post-translational modifications with important physiological functions (6), we aimed at identifying the exact sites of PARP1-mediated ADP-ribosylation. To this end, we first generated a series of histone tail deletion mutants to test in ADP-ribosylation

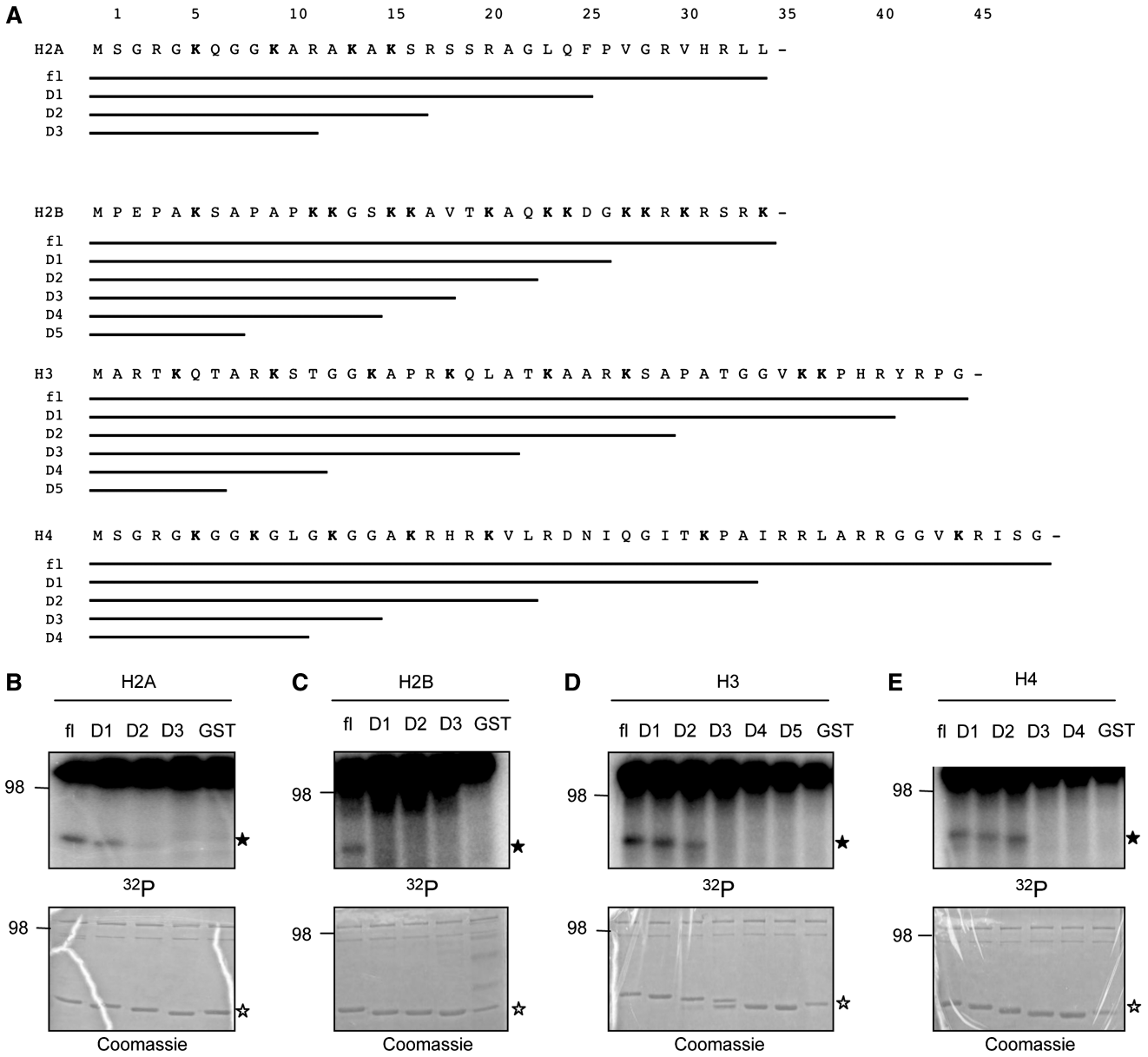


Figure 2. Confining the regions of putative ADP-ribose acceptor sites. (**A**) Schematic representation of the deletion strategy for GST-histone tails to identify the minimal ADP-ribosylated domain. (**B–E**) *Trans*-ADP-ribosylation of the indicated GST-histone deletion mutants by PARP1 with 100 nM ^{32}P-NAD^{+}.

assays (Figure 2A). Successive shortening of the histone tails invariably resulted in a loss of PARP1-mediated ADP-ribosylation and defined for each histone the region comprising putative ADP-ribose acceptor sites (Figure 2B–E).

To directly identify the ADP-ribosylated amino acids within the histone tails by mass spectrometry, we used synthetic peptides covering the regions identified by our deletion strategy. We incubated these peptides with PARP1 in the presence of 100–500 μM NAD^{+}, stopped the reactions by addition of 3AB and subsequently treated the poly(ADP-ribosyl)ated peptides with ADP-ribosylhydrolase 3 (ARH3). ARH3 is known to possess PARG-like ADP-ribose glycohydrolase activity, which hydrolyzes ester linkages between ADP-ribose units (31). Since no negatively charged amino acids (E or D), which would allow the formation of an ester linkage, were present in the polypeptides (except for H2B E35), we rationalized that treatment of the modified polypeptides with ARH3 would leave the first ADP-ribose unit bound to the peptide. The ADP-ribosylated peptides were acetone precipitated and analyzed by liquid-chromatography coupled mass-spectrometry. In the presence of PARP1 and NAD^{+}, the attachment of a single ADP-ribose unit resulted in a mass shift of 541 Dalton (Figure 3A–D). Fragmentation of ADP-ribosylated peptides with conventional collision induced dissociation (CID) fragmentation

technique completely removed the ADP-ribose moiety from the peptides, not allowing the identification of specific amino-acid acceptor sites (data not shown). In contrast, analysis of the modified peptides by electron transfer dissociation (ETD) resulted in an almost complete fragmentation of the multiply charged histone peptides, as indicated by the presence of c- and z-ions, which represent N- or C-terminal fragment ions, respectively (Figure 3A–D, Supplementary Figure S2E). Fragmentation of the mono(ADP-ribosyl)ated H2A peptide revealed specific ADP-ribosylation of K13, while H2B was mainly ADP-ribosylated at K30 (Figure 3A and B). H3 was ADP-ribosylated at K27 and K37 (Figure 3C and Supplementary Figure S2E). For H4, mass spectrometric analysis identified K16 to be ADP-ribosylated by PARP1 (Figure 3D). Control reactions performed in the presence of 500 μM NAD^+ but without PARP1 or in the presence of 500μM ADP-ribose and PARP1 did not result in specific ADP-ribosylation (data not shown). The identified sites of PARP1-mediated enzymatic ADP-ribosylation represent two known sites of frequent histone modification (H3K27 and H4K16) as well as novel modification sites (H2AK13, H2BK30 and H3K37). To verify the mass spectrometric data for one histone, the H4 tail was mutated at K16 to alanine and tested for ADP-ribosylation by PARP1. The mutated H4 tail showed severely reduced ADP-ribosylation in comparison to the wild-type H4 tail (Figure 3E, filled asterisk). The reduction of ADP-ribosylation was not due to a reduced interaction of PARP1 with the mutated H4 tail, since wild-type and mutated histone tail fusion proteins were able to interact with PARP1 to comparable levels (Figure 3F).

Modeling of the histone H4 tail reveals that R17 is critical for the interaction with the catalytic domain of PARP1

In order to test computationally, whether the tail of H4 could enter the catalytic cleft of PARP1, automatic docking followed by explicit solvent MD simulations were performed with the crystal structure from chicken PARP1 (PDB ID: 1A26), which uses the sequence numbering of human PARP1 (see 'Materials and methods' section). The MD simulations indicate that the H4 tetrapeptide segment AKRH (aa 15–18) binds in an extended conformation to the catalytic domain of PARP1 (Figure 4A and B). Two stable salt bridges are observed in all MD runs: between H4K16 and PARP1 Glu988 in the catalytic cleft, and between H4R17 and PARP1 Glu756 (Asp756 in human PARP1) on a loop at the entrance of the cleft. These intermolecular salt bridges lock H4 into the catalytic domain of PARP1 like two stretched arms holding two points far away from each other. In all MD runs, a single water molecule inserts between the amino group of K16 and the carboxy group of Glu988 in the first 15 ns and remains between these two charged groups until the end of the MD simulations of the tetrapeptide (Supplementary Figure S3). This water molecule occupies the same position as the water molecule that is close to Glu988 in the X-ray structure of PARP1 (water 37 in PDB code 1A26). There are two additional side chains interactions: a stacking interaction between the imidazole of H18 and the amide group of Gln759 is almost always present in all MD runs, while the hydrogen bond between the side chains of R19 and Asn906 is not very stable. In contrast to the aforementioned intermolecular salt bridges and hydrogen bonds, the K20 side chain is always exposed to solvent and very flexible. Furthermore, the backbone polar groups of the histone do not seem to be involved in hydrogen bonds with PARP1. Importantly, in all MD simulations both the N-terminal and C-terminal methyl groups point towards the solvent, which would allow the rest of the H4 polypeptide chain to position itself close to the surface of PARP1 without steric clashes. Moreover, the MD runs with the H4 tetra- and octapeptide (aa 15–22) converge towards a common extended structure of the AKRH segment with the same side chain interactions. The convergence of multiple MD simulations and the agreement with the experimental results indicate that the binding mode obtained by docking and explicit water MD is reliable. To gain insight in the putative initiation step of ADP-ribosylation, the 10 ns snapshot of the MD simulation with the H4 tetrapeptide was used for docking NAD^+ into the donor site as previously published (32,33). Before docking, the nicotinamide was manually removed (Figure 4C), which mimics the NADase activity of PARP1 and creates a reactive C1-atom of the ADP-ribose that is suggested to react with the substrate amino acid (19,34,35). Interestingly, the C1-atom of the ADP-ribose is only 3.7 Å away from the ε-amino group of H4K16 and 7.8 Å from the ε-amino group of Lys903. Moreover, the distance between the catalytic active Glu988 carboxy group and the ε-amino group of H4K16 is only 3.0 Å, which would potentially allow covalent ADP-ribosylation of H4K16 (Figure 4C).

To test experimentally whether an arginine close to H4K16 is required for the interaction with PARP1, we mutated R17 (H4R17A) and analyzed the association with and the modification by PARP1. GST-pulldown experiments with recombinant PARP1 revealed that the interaction between PARP1 and the mutated H4 tail fusion protein was reduced (Figure 4D), as well as its ADP-ribosylation by PARP1 (Figure 4E). In agreement with the MD simulation and the mass spectrometry data, an H4K16A/R17A double mutant was completely defective for PARP1-mediated ADP-ribosylation (Figure 4F). Since H4R17 was suggested by molecular modeling to interact with Asp756 of PARP1, we mutated Asp756 into a lysine (which is the corresponding amino acid at this position in PARP2) and tested this mutant for its ADP-ribosylation properties. The PARP1 mutant exhibited no defect in auto(ADP-ribosyl)ation (Supplementary Figure S2F), indicating that Asp756 is not essential for automodification. This is consistent with the fact that also PARP2 is able to modify itself, although it contains a lysine at the corresponding position. Interestingly, however, the PARP1 mutant was impaired *in trans*-ADP-ribosylation of full-length histones (Figure 4G) and in the labeling of the H4 (1–22) peptide (Supplementary Figure S1G), confirming the MD-based

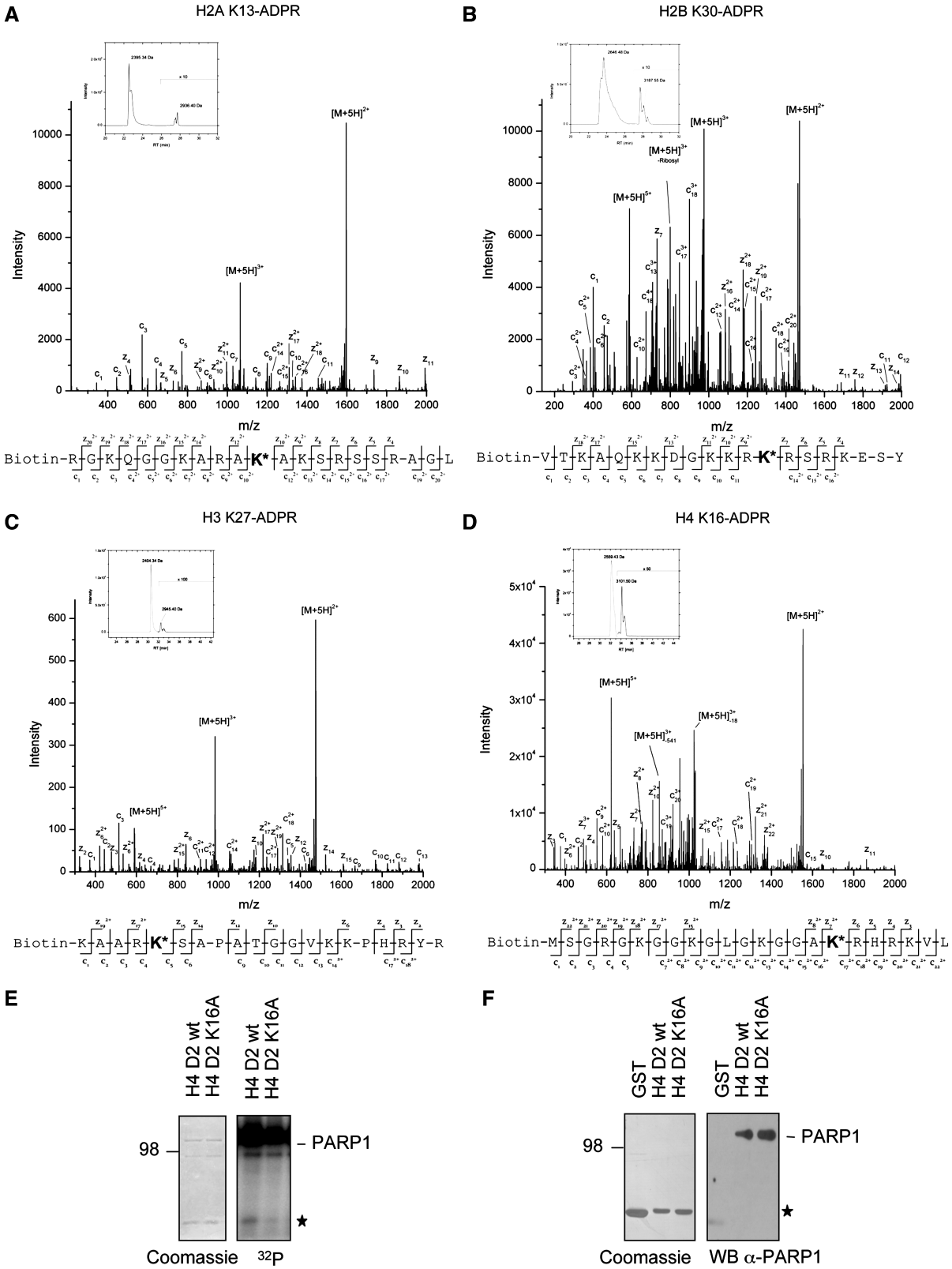


Figure 3. Mass spectrometric analysis of ADP-ribosylated histone peptides. (**A**) Extracted ion chromatogram of the biotin tagged H2A (aa 3–23) peptide incubated with 500 μM NAD^+ and PARP1. The precursor masses of unmodified H2A peptide (2395.34 Da) and ADP-ribosylated H2A peptide (2936.40 Da) were plotted in a range of 10 ppm over time. ETD fragment spectrum of quintuply charged precursor ion of ADP-ribosylated H2A peptide (638.51 m/z) at K13, as indicated by the sequence plot. The spectrum shows next to the two major peaks of unfragmented charge reduced precursor ions $(M+5H)^{n+}$ an almost complete series of N-terminal and C-terminal fragment ions (c-ions, z-ions, respectively). (**B**) Extracted ion chromatogram of the biotin tagged H2B (aa 18–37) peptide incubated with 500 μM NAD^+ and PARP1 as in (A). ETD fragment spectrum of ADP-ribosylated H2B peptide (797.89 m/z) at K30, indicated by the sequence plot. (**C**) Extracted ion chromatogram of the biotin tagged H3 (aa 23–42) peptide incubated with PARP1 as in (A). ETD fragment spectrum of ADP-ribosylated H3 peptide (590.29 m/z) at K27, indicated by the sequence plot. (**D**) Extracted ion chromatogram of the biotin tagged H4 (aa 1–22) peptide incubated with 100 μM NAD^+ and PARP1. ETD fragment spectrum of ADP-ribosylated H4 peptide (621.30 m/z) at K16, as indicated by the sequence plot. (**E**) *Trans*-ADP-ribosylation of the GST-H4 histone tail wild-type or K16A mutant by PARP1. (**F**) GST-pulldown of wild-type and mutated GST-histone H4 tails with recombinant PARP1. The GST-histone tails are marked by an asterisk.

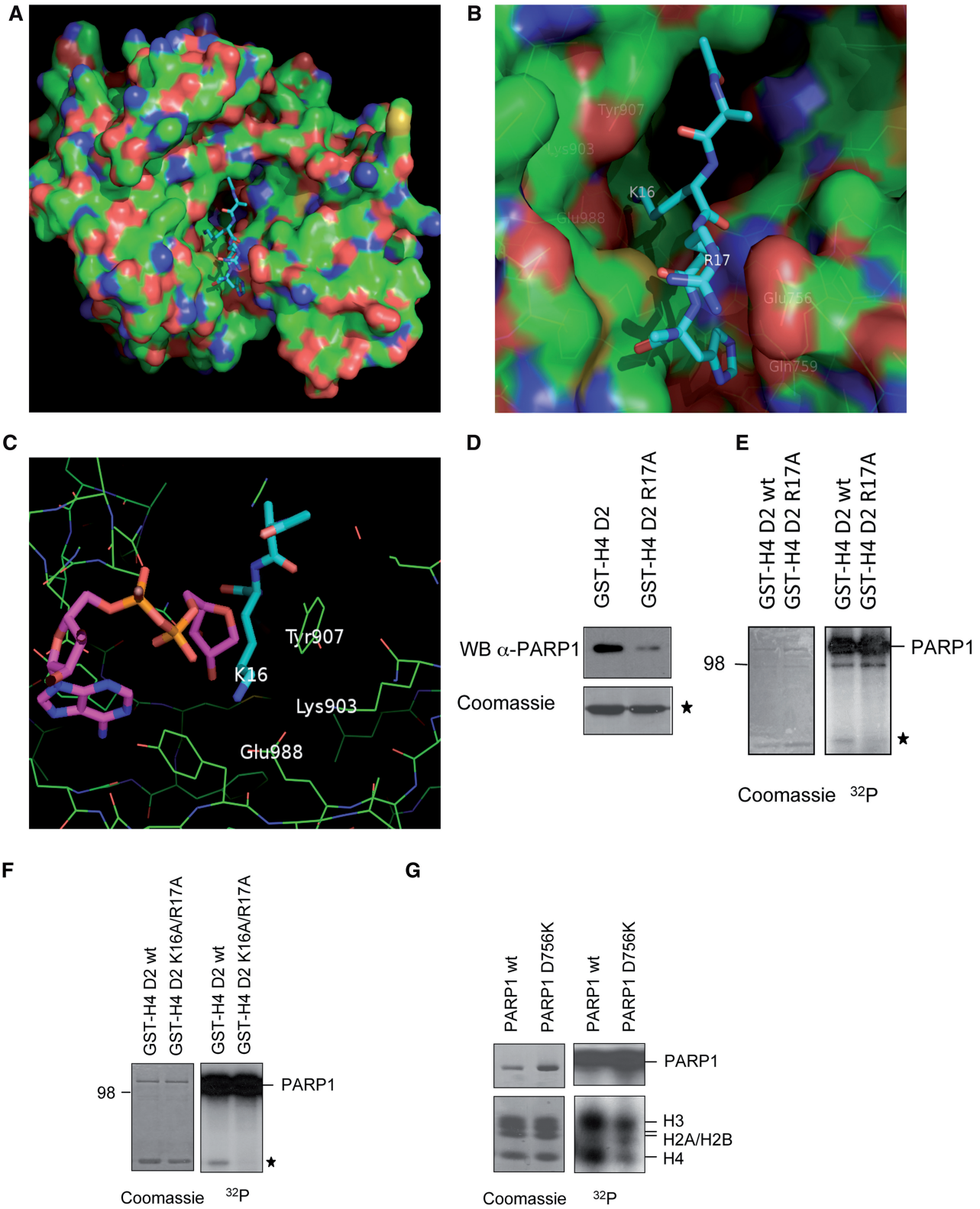


Figure 4. Histone H4 interacts with PARP1 by salt bridges with acidic residues in the catalytic domain. (**A**) Representative snapshot of the binding mode saved after 10 ns MD simulation of the H4 tetrapeptide AKRH. (**B**) Enlarged view of the catalytic cleft for the same snapshot as in (A). Amino acids in close proximity of the H4 peptide are highlighted. PARP1 and the H4 tetrapeptide are shown in surface render and sticks, respectively. The surface is colored according to atomic elements with carbon, oxygen and nitrogen in green, red, and blue, respectively. Carbon atoms of the H4 tetrapeptide are in cyan. (**C**) ADP-ribose was docked into the donor-site of PARP1 catalytic domain after 10 ns MD simulation of the H4 tetrapeptide. Amino acids in close proximity of the H4 peptide are highlighted. The orientation is rotated by about 180°C with respect to (A,B). (**D**) GST-pulldown of wild-type and mutated GST-histone H4 tail with recombinant PARP1 and subsequent western blot with anti-PARP1 antibody. GST-histone tails are indicated by an asterisk. (**E** and **F**) *Trans*-ADP-ribosylation of wild-type and mutated GST-H4 tails with PARP1 and radiolabeled NAD^+. Coomassie stains of the input and autoradiographies are shown. (**G**) *Trans*-ADP-ribosylation of calf-thymus extracted histones by PARP1 wild-type or PARP1 D756K mutant as in Figure 4A.

prediction of the importance of this residue for stabilization of the peptide in the catalytic cleft and subsequent *trans*(ADP-ribosyl)ation of H4.

Acetylation of K16 inhibits ADP-ribosylation of histone H4

Acetylation of H4K16 occurs frequently in eukaryotic cells and has been correlated with chromatin decompaction (6). If H4K16 was indeed an acceptor site for PARP1-mediated ADP-ribosylation, acetylation at that site should impair ADP-ribosylation. In order to test this hypothesis, we employed an H4 peptide (aa 1–22) chemically acetylated at K16. LC-coupled mass spectrometry of the H4K16ac peptide revealed that PARP1 was not any longer able to induce ADP-ribosylation of the acetylated peptide above background (Figure 5A). Consistent with this result, PARP1 mediated ADP-ribosylation of both peptides (unmodified and acetylated) with radiolabeled NAD^+, followed by purification over a microvolume-C18 reversed phase column and subsequent measurement of incorporated radiolabeled NAD^+, provided evidence that ADP-ribosylation of the acetylated peptide was severely reduced (Figure 5B). These results confirm that H4K16 is modified by PARP1 and show that acetylation of K16 severely impairs ADP-ribosylation of the H4 peptide. Together, our results led to the identification of ADP-ribose acceptor sites within the amino-terminal tails of the four core histones (Figure 5C) and imply important cross-talks with other histone modifications such as acetylation or methylation.

DISCUSSION

Here, we provide evidence that PARP1 covalently modifies the tails of all four core histones. We identified H2AK13, H2BK30, H3K27, H3K37 and H4K16 as specific target sites for PARP1-mediated ADP-ribosylation. Our conclusions are based on several observations: (i) mass spectrometric analyses of PARP1-mediated ADP-ribosylated peptides, (ii) loss of function experiments by site directed mutagenesis of the putative acceptor sites in recombinant histone tail fusion proteins, (iii) cross-talk of acetylation and ADP-ribosylation at the identified acceptor site in H4 and, finally, (iv) prediction of the interaction between the histone H4 tail and PARP1 by MD and subsequent confirmation with mutated proteins.

Nearly 20 years ago, ADP-ribose acceptor sites were found in histones by biochemical approaches. Several laboratories identified glutamic acid residues in histone H1 and histone H2B to be modified when they incubated chromatin from rat liver with radioactive NAD^+ (11,13–15). At that time, no other PARP family member had been identified yet, and no knockout- or knockdown-system was available. Thus, it is possible that PARP1, other PARP-family members or even unrelated NAD^+ consuming enzymes were responsible for the modification at the identified glutamates. In fact, we could show here by mutational analyses, that E2 of H2B is dispensable for PARP1-mediated ADP-ribosylation and that additional amino-acid residues are acceptors for ADP-ribose moieties. Consistent with this, we identify lysines in the amino terminal histone tails and in particular lysine 13 of H2A, lysine 30 of H2B, lysines 27 and 37 of histone H3, as well as lysine 16 of histone H4 as target sites for enzymatic ADP-ribosylation by PARP1, both by mass spectrometry and amino-acid substitution. Therefore, we propose the ADP-ribosylation of the ε-amino group of lysines by PARP1 as a new canonical histone tail modification.

Remarkably, explicit solvent MD revealed that the tetra- and octapeptides of H4 interact strongly with specific amino-acid side chains of the catalytic cleft of PARP1, suggesting that specific binding of a relatively short segment of H4 is sufficient to allow poly(ADP-ribosyl)ation of the histone tail. The positively charged amino acid at the +1 position of the ADP-ribosylated residue formed a salt bridge with Glu756 of chicken PARP1, which corresponds to Asp756 in human PARP1. In contrast, the corresponding amino acid in PARP2 is a lysine at position 312. Although the catalytic domains of PARP1 and PARP2 are highly similar (18), the substrate specificity of those enzymes might be regulated by subtle differences in the catalytic cleft. This could explain why PARP2 does not modify histones to a detectable extent at least *in vitro* (Figure 1A, C and Supplementary Figure S2B).

Application of novel mass spectrometry techniques allowed us to identify distinct amino acids as acceptors of ADP-ribose. We took advantage of the ETD technique (36), which allows the fragmentation of highly charged peptides, leaving most post-translational modifications intact. Recent publications describe the technical basis for the fragmentation of chemically ADP-ribosylated peptides by electron capture dissociation (ECD) (37) and the closely related ETD (38). It is noteworthy, that, in contrast to other reports, we observed partial fragmentation of the ADP-ribose at the phosphodiester bond by application of ETD, as revealed by the presence of a m/z 348 ion. However, conventional CID mass spectrometry of ADP-ribosylated H4 peptide did not reveal any ADP-ribose acceptor sites, since the ADP-ribose was cleaved off from the peptide during fragmentation. The commonly used CID, instead of ETD, might thus explain why numerous efforts to identify ADP-ribosylated residues failed in the past. Consequently, we would strongly recommend ETD as standard technique to detect ADP-ribosylated peptides. Of note, a previous study employing CID failed to detect ADP-ribosylated lysine residues in the catalytic PARP1 mutant E988Q (39). In summary, ETD can be expected to facilitate future investigations on ADP-ribosylated peptides, opening new opportunities to screen for ADP-ribosylated residues in a systems-biology setup.

Since poly(ADP-ribosyl)ated peptides cannot be detected by MS and PARP1 mainly attaches poly(ADP-ribose) to target proteins, we removed the poly(ADP-ribose) with ARH3, which degrades poly(ADP-ribose). However, this treatment was rather inefficient (data not shown), which could partly explain the observed low mono(ADP-ribosyl)ation of the histone

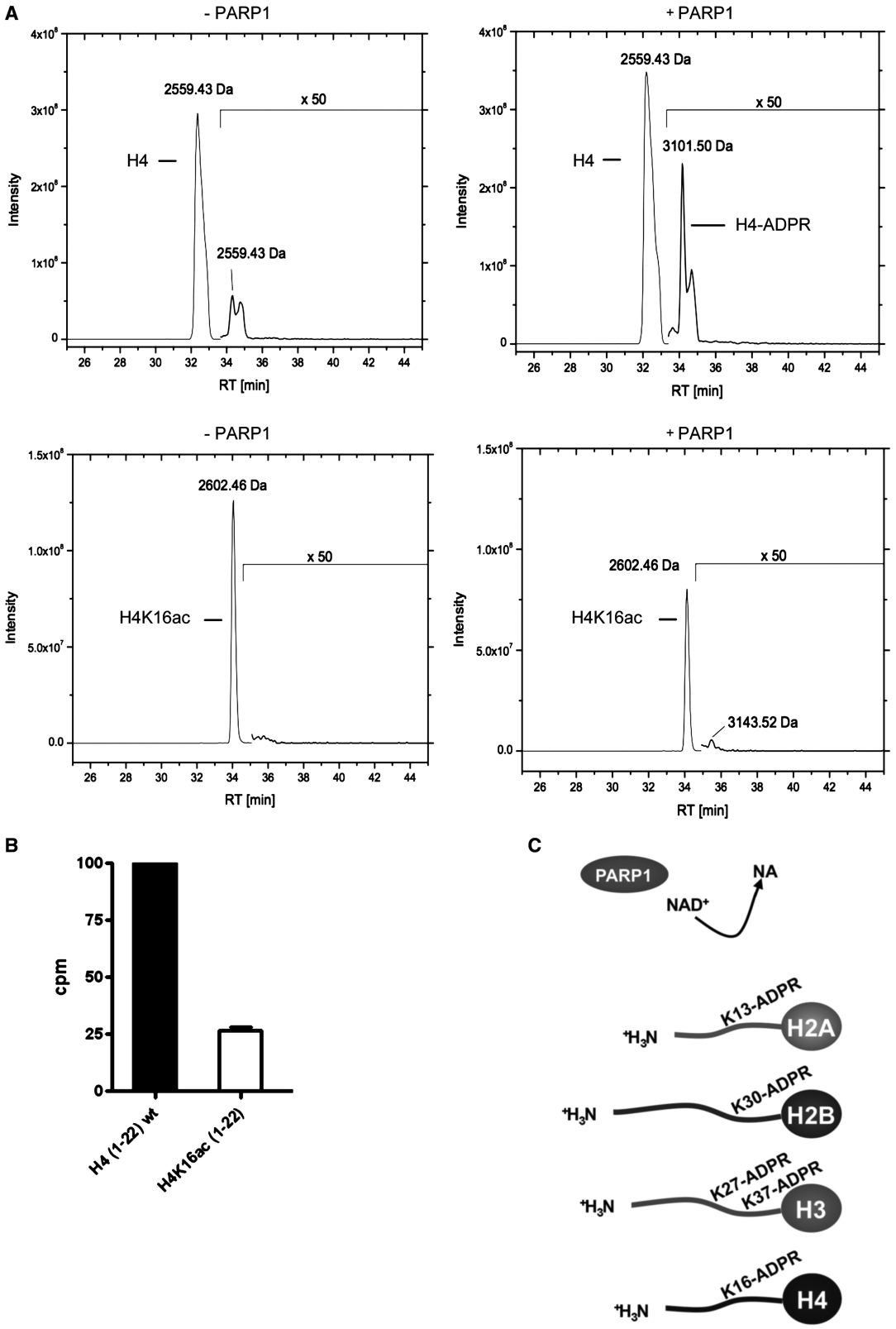


Figure 5. ADP-ribosylation of the H4 peptide is impaired by H4K16 acetylation. (**A**) Elution profile of biotinylated H4 peptide (aa 1–22) and biotinylated H4K16ac peptide (aa 1–22) incubated with 100 μM NAD^+ for 15 min without PARP1 (–PARP1) or in the presence of PARP1 (+PARP1) and subsequent ARH3 treatment. Acetone precipitated peptides were analyzed by LC–MS/MS using a C18 reversed-phase column and subsequent detection by mass spectrometry. (**B**) Histone H4 (aa 1–22) and acetylated H4K16ac (1–22) peptides were ADP-ribosylated with PARP1 for 15 min at 30°C with 100 nM ^{32}P-NAD^+. The peptides were purified by microvolume-C18 reversed phase columns, eluted into scintillation liquid and counted for incorporated ^{32}P. Relative increase of counts per minutes was calculated over background (peptides added after termination of the reaction by 3AB). (**C**) Overview of the identified ADP-ribose acceptor sites within the amino-terminal core histone tails.

peptides. Moreover, we cannot exclude that PARP1 modifies additional residues, which were not identified by these mass spectrometric analyses.

Our data provide strong evidence that PARP1-mediated ADP-ribosylation of histones occurs as post-translational modification at distinct lysine residues within the amino-terminal basic tails. From a chemical perspective, modification of a lysine residue by ADP-ribose results in an unstable Schiff base, which can undergo an Amadori rearrangement to form a stable ketoamine (19,40). It will be interesting to investigate whether histone lysine ADP-ribosylation can be reversed by a previously identified but still poorly characterized ADP-ribosyl protein lyase (41). Since the attachment of ADP-ribose not only neutralizes the positive charge of the amino-acid side chain, but instead reverses it into a negative charge, the functional consequences of lysine ADP-ribosylation can be assumed to be even more drastic than those of other modifications, such as acetylation. Therefore, the possible effects on chromatin architecture, histone dynamics, histone degradation, and histone variant incorporation may be dramatic. Possibly, ADP-ribosylation of histones interferes with other post-translational modifications of the histone tails. For example, H3K27 is methylated by EZH2 (enhancer of zeste homolog 2), which is in the polycomb group complex that is involved in maintenance of the inactive X-chromosome (42). Interestingly, PARP1 was demonstrated to participate in the maintenance of X-chromosome silencing as well (43). On the other hand, the amino-terminal tail of histone H4 was shown to be required for chromatin fiber formation, since the positively charged stretch between K16 and K20 makes internucleosomal contacts to two acidic patches on the carboxy-terminal α-helices of histone H2A (44). In addition to its function for chromatin topology, the stretch between K16 and K20 is required for the interaction with various non-histone modulators. For example, the ISWI-containing ATP dependent chromatin remodeler ACF solely engages histone H4, but is repelled, if H4K16 is acetylated (45,46). Furthermore, the chromatin remodeler Alc1 was shown to require the K16 to K20 stretch of H4 for its activity (47). Interestingly, recent reports provide evidence that the ATPase activity of Alc1 is highly stimulated by binding to poly(ADP-ribosyl)ated PARP1 (47,48). Whether ADP-ribosylated H4 would activate Alc1, remains to be investigated. Another intriguing possibility of how histone tail ADP-ribosylation could affect chromatin function is implied by a recent study showing that macrodomain-containing histone variants specifically bind to poly(ADP-ribose) generated after DNA damage (49). Using biochemical, crystallographic and state-of-the-art imaging techniques, it was shown that macroH2A1.1 senses PARP1 activation and directly binds poly (ADP-ribose) to cause a transient compaction of the chromatin. It will be interesting to investigate whether macrodomains preferentially bind to automodified PARP1 or also function as 'readers' of poly (ADP-ribosyl)ated histone tails.

Taken together, the work presented here sheds new light on a well known but neglected histone modification and builds the basis for future investigations exploring the function of histone lysine ADP-ribosylation in chromatin dynamics, transcription, DNA repair signaling and other nuclear processes influenced by histone modifications.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors are grateful to Timothy Richmond (ETH Zurich, Switzerland) and Achim Leutz (Max-Delbrück-Centrum for Molecular Medicine, Berlin, Germany) for the generous provision of reagents and sequences. Brighton Samatanga (University of Zurich, Switzerland) is acknowledged for his technical support. The MD stimulations were carried out on the Schroedinger computer cluster at the University of Zurich. The authors thank Raffaella Santoro (University of Zurich, Institute of Veterinary Biochemistry and Molecular Biology) for critical and constructive comments.

FUNDING

Funding for open access charge: Swiss National Science Foundation (Grant 31-122421 to S.M. and M.A.); The Kanton of Zurich (to M.O.H.).

Conflict of interest statement. None declared.

REFERENCES

1. Kornberg,R.D. (1974) Chromatin structure: a repeating unit of histones and DNA. *Science*, **184**, 868–871.
2. Davey,C.A., Sargent,D.F., Luger,K., Maeder,A.W. and Richmond,T.J. (2002) Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. *J. Mol. Biol.*, **319**, 1097–1113.
3. Luger,K., Mäder,A.W., Richmond,R.K., Sargent,D.F. and Richmond,T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature*, **389**, 251–260.
4. Campos,E. and Reinberg,D. (2009) Histones: annotating chromatin. *Annu. Rev. Genet.*, **43**, 559–599.
5. Angelov,D., Vitolo,J.M., Mutskov,V., Dimitrov,S. and Hayes,J.J. (2001) Preferential interaction of the core histone tail domains with linker DNA. *Proc. Natl Acad. Sci. USA*, **98**, 6599–6604.
6. Kouzarides,T. (2007) Chromatin modifications and their function. *Cell*, **128**, 693–705.
7. Ueda,K., Omachi,A., Kawaichi,M. and Hayaishi,O. (1975) Natural occurrence of poly(ADP-ribosyl) histones in rat liver. *Proc. Natl Acad. Sci. USA*, **72**, 205–209.
8. Otake,H., Miwa,M., Fujimura,S. and Sugimura,T. (1969) Binding of ADP-ribose polymer with histone. *J. Biochem.*, **65**, 145–146.
9. Adamietz,P., Bredehorst,R. and Hilz,H. (1978) ADP-ribosylated histone H1 from HeLa cultures. Fundamental differences to (ADP-ribose)n-histone H1 conjugates formed into vitro. *Eur. J. Biochem.*, **91**, 317–326.
10. Minaga,T., Romaschin,A.D., Kirsten,E. and Kun,E. (1979) The in vivo distribution of immunoreactive larger than tetrameric polyadenosine diphosphoribose in histone and non-histone protein fractions of rat liver. *J. Biol. Chem.*, **254**, 9663–9668.

11. Ogata,N., Ueda,K. and Hayaishi,O. (1980) ADP-ribosylation of histone H2B. Identification of glutamic acid residue 2 as the modification site. *J. Biol. Chem.*, **255**, 7610–7615.
12. Boulikas,T. (1988) At least 60 ADP-ribosylated variant histones are present in nuclei from dimethylsulfate-treated and untreated cells. *EMBO J.*, **7**, 57–67.
13. Burzio,L.O., Riquelme,P.T. and Koide,S.S. (1979) ADP ribosylation of rat liver nucleosomal core histones. *J. Biol. Chem.*, **254**, 3029–3037.
14. Riquelme,P.T., Burzio,L.O. and Koide,S.S. (1979) ADP ribosylation of rat liver lysine-rich histone in vitro. *J. Biol. Chem.*, **254**, 3018–3028.
15. Ogata,N., Ueda,K., Kagamiyama,H. and Hayaishi,O. (1980) ADP-ribosylation of histone H1. Identification of glutamic acid residues 2, 14, and the COOH-terminal lysine residue as modification sites. *J. Biol. Chem.*, **255**, 7616–7620.
16. Hassa,P.O., Haenni,S.S., Elser,M. and Hottiger,M.O. (2006) Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol. Mol. Biol. Rev.*, **70**, 789–829.
17. Quénet,D., El Ramy,R., Schreiber,V. and Dantzer,F. (2009) The role of poly(ADP-ribosyl)ation in epigenetic events. *Int. J. Biochem. Cell Biol.*, **41**, 60–65.
18. Amé,J.C., Rolli,V., Schreiber,V., Niedergang,C., Apiou,F., Decker,P., Muller,S., Höger,T., Ménissier-de Murcia,J. and de Murcia,G. (1999) PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. *J. Biol. Chem.*, **274**, 17860–17868.
19. Altmeyer,M., Messner,S., Hassa,P.O., Fey,M. and Hottiger,M.O. (2009) Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites. *Nucleic Acids Res.*, **37**, 3723–3738.
20. Haenni,S.S., Hassa,P.O., Altmeyer,M., Fey,M., Imhof,R. and Hottiger,M.O. (2008) Identification of lysines 36 and 37 of PARP-2 as targets for acetylation and auto-ADP-ribosylation. *Int. J. Biochem. Cell Biol.*, **40**, 2274–2283.
21. Hassa,P.O., Haenni,S.S., Buerki,C., Meier,N.I., Lane,W.S., Owen,H., Gersbach,M., Imhof,R. and Hottiger,M.O. (2005) Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription. *J. Biol. Chem.*, **280**, 40450–40464.
22. Hassa,P.O., Buerki,C., Lombardi,C., Imhof,R. and Hottiger,M.O. (2003) Transcriptional coactivation of nuclear factor-kappaB-dependent gene expression by p300 is regulated by poly(ADP)-ribose polymerase-1. *J. Biol. Chem.*, **278**, 45145–45153.
23. Kale,L., Skeel,R., Bhandarkar,M., Brunner,R., Gursoy,A., Krawetz,N., Phillips,J., Shinozaki,A., Vraradarajan,K. and Schulten,K. (1999) NAMD2: greater scalability for parallel molecular dynamics. *J. Comput. Phys.*, **151**, 283–312.
24. MacKerell,A.D., Bashford,D., Bellott,M., Dunbrack,R.L., Evanseck,J.D., Field,M.J., Fischer,S., Gao,J., Guo,H., Ha,S. *et al.* (1998) All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J . Phys. Chem. B*, **102**, 3586–3616.
25. Jorgensen,W.L., Chandrasekhar,J. and Madura,J.D. (1983) Comparison of simple potential functions for simulating liquid water. *J . Phys. Chem. B*, **79**, 926–936.
26. Darden,T., York,D. and Pedersen,L. (1993) Particle mesh Ewald: An N.log(N) method for Ewald sums in large systems. *J. Chem. Phys.*, **98**, 10089–10092.
27. Brooks,B.R., Brooks,C.L., Mackerell,A.D., Nilsson,L., Petrella,R.J., Roux,B., Won,Y., Archontis,G., Bartels,C., Boresch,S. *et al.* (2009) CHARMM: the biomolecular simulation program. *J. Comput. Chem.*, **30**, 1545–1614.
28. Seeber,M., Cecchini,M., Rao,F., Settanni,G. and Caflisch,A. (2007) Wordom: a program for efficient analysis of molecular dynamics simulations. *Bioinformatics*, **23**, 2625–2627.
29. Jump,D.B., Sudhakar,S., Tew,K.D. and Smulson,M. (1980) Probes to study the effect of methyl nitrosourea on ADP-ribosylation and chromatin structure at the subunit level. *Chem. Biol. Interact.*, **30**, 35–51.
30. Pleschke,J.M., Kleczkowska,H.E., Strohm,M. and Althaus,F.R. (2000) Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. *J. Biol. Chem.*, **275**, 40974–40980.
31. Oka,S., Kato,J. and Moss,J. (2006) Identification and characterization of a mammalian 39-kDa poly(ADP-ribose) glycohydrolase. *J. Biol. Chem.*, **281**, 705–713.
32. Ruf,A., Rolli,V., de Murcia,G. and Schulz,G.E. (1998) The mechanism of the elongation and branching reaction of poly(ADP-ribose) polymerase as derived from crystal structures and mutagenesis. *J. Mol. Biol.*, **278**, 57–65.
33. Ruf,A., de Murcia,G. and Schulz,G.E. (1998) Inhibitor and NAD+ binding to poly(ADP-ribose) polymerase as derived from crystal structures and homology modeling. *Biochemistry*, **37**, 3893–3900.
34. Alvarez-Gonzalez,R., Pacheco-Rodriguez,G. and Mendoza-Alvarez,H. (1994) Enzymology of ADP-ribose polymer synthesis. *Mol. Cell Biochem.*, **138**, 33–37.
35. Bürkle,A. (2005) Poly(ADP-ribose). The most elaborate metabolite of NAD+. *FEBS J.*, **272**, 4576–4589.
36. Syka,J.E.P., Coon,J.J., Schroeder,M.J., Shabanowitz,J. and Hunt,D.F. (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl Acad. Sci. USA*, **101**, 9528–9533.
37. Hengel,S.M., Shaffer,S.A., Nunn,B.L. and Goodlett,D.R. (2009) Tandem mass spectrometry investigation of ADP-ribosylated kemptide. *J. Am. Soc. Mass Spectrom*, **20**, 477–483.
38. Zee,B.M. and Garcia,B.A. (2010) Electron transfer dissociation facilitates sequencing of adenosine diphosphate-ribosylated peptides. *Anal. Chem.*, **82**, 28–31.
39. Tao,Z., Gao,P. and Liu,H. (2009) Identification of the ADP-ribosylation sites in the PARP-1 automodification domain: analysis and implications. *J. Am. Chem. Soc.*, **131**, 14258–14260.
40. Cervantes-Laurean,D., Jacobson,E.L. and Jacobson,M.K. (1996) Glycation and glycoxidation of histones by ADP-ribose. *J. Biol. Chem.*, **271**, 10461–10469.
41. Oka,J., Ueda,K., Hayaishi,O., Komura,H. and Nakanishi,K. (1984) ADP-ribosyl protein lyase. Purification, properties, and identification of the product. *J. Biol. Chem.*, **259**, 986–995.
42. Heard,E. (2005) Delving into the diversity of facultative heterochromatin: the epigenetics of the inactive X chromosome. *Curr. Opin. Genet. Dev.*, **15**, 482–489.
43. Nusinow,D.A., Hernández-Muñoz,I., Fazzio,T.G., Shah,G.M., Kraus,W.L. and Panning,B. (2007) Poly(ADP-ribose) polymerase 1 is inhibited by a histone H2A variant, MacroH2A, and contributes to silencing of the inactive X chromosome. *J. Biol. Chem.*, **282**, 12851–12859.
44. Zhou,J., Fan,J.Y., Rangasamy,D. and Tremethick,D.J. (2007) The nucleosome surface regulates chromatin compaction and couples it with transcriptional repression. *Nat. Struct. Mol. Biol.*, **14**, 1070–1076.
45. Clapier,C.R., Längst,G., Corona,D.F., Becker,P.B. and Nightingale,K.P. (2001) Critical role for the histone H4 N terminus in nucleosome remodeling by ISWI. *Mol. Cell. Biol.*, **21**, 875–883.
46. Shogren-Knaak,M., Ishii,H., Sun,J.-M., Pazin,M.J., Davie,J.R. and Peterson,C.L. (2006) Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science*, **311**, 844–847.
47. Ahel,D., Horejsi,Z., Wiechens,N., Polo,S., Garcia-Wilson,E., Ahel,I., Flynn,H., Skehel,M., West,S., Jackson,S. *et al.* (2009) Poly(ADP-ribose)-dependent regulation of DNA repair by the chromatin remodeling enzyme ALC1. *Science*, **325**, 1240–1243.
48. Gottschalk,A., Timinszky,G., Kong,S., Jin,J., Cai,Y., Swanson,S., Washburn,M., Florens,L., Ladurner,A., Conaway,J. *et al.* (2009) Poly(ADP-ribosyl)ation directs recruitment and activation of an ATP-dependent chromatin remodeler. *Proc. Natl Acad. Sci. USA*, **106**, 13770–13774.
49. Timinszky,G., Till,S., Hassa,P., Hothorn,M., Kustatscher,G., Nijmeijer,B., Colombelli,J., Altmeyer,M., Stelzer,E., Scheffzek,K. *et al.* (2009) A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation. *Nat. Struct. Mol. Biol.*, **16**, 923–929.

Supplementary Methods

Cell culture, transfection of siRNA and overexpression of PARP1

HEK293T cells were grown in DMEM Glutamax-1 (Invitrogen), supplemented with 10% (v/v) fetal calf serum (Invitrogen) and 50 units/ml penicillin and 50 μg/ml streptomycin (Sigma). Cells were grown in 5% CO_2 and 37°C in a humidified incubator. HEK293T cells were transfected with RNAi-max (Invitrogen) and siRNA directed against PARP1 (Qiagen, Cat.No. S102662989) or control siRNA (Qiagen, Cat.No. S103650318) for 48 hours. Overexpression of PARP1 was performed by transfection of HEK293T cells by standard Calcium-Phosphate precipitation method with a pcDNA3-HA-PARP1 expression vector or an empty vector control, respectively. 8 hours after transfection the medium was replaced and the cells were grown for another 20 hours before they were harvested.

ADP-ribosylation of isolated nuclei

Nuclei were isolated from 5x 10^6 HEK293T cells by the addition of cold hypotonic lysis buffer (5 mM HEPES pH 7.4, 0.5% NP-40, 85 mM KCl, 1 μg/ul Pepstatin, Leupetin, Bestatin). After 2 minutes of incubation, the nuclei were centrifuged for 4 min at 7000g and washed twice with suspension buffer (33.3 mM Tris-HCl pH 7.8, 40 mM $MgCl_2$, 1 μg/ul Pepstatin, Leupetin, Bestatin). The pellet was resuspended in permeabilization buffer (38.3 mM Tris-HCl pH 7.8, 42.1 mM $MgCl_2$, 0.53 mM EDTA, 13.9 mM β-Mercaptoethanol, 1 μg/ul Pepstatin, Leupetin, Bestatin), supplemented with 400 μM etheno-NAD^+ (Sigma Aldrich) or 4 mM PJ34 (Enzo Life Science) and incubated for 20 min at 37°C at 900 rpm in a rotator. After centrifugation the pellet was resuspended in SDS-lysis buffer and run on a 18% SDS-PAGE. Western blotting was performed with anti Ig4 antibody hybridoma serum (kindly provided by Dr. R. Santella, Columbia University, USA) in a vacuum blot apparatus (Millipore SNAP i.d).

Supplementary Figures

Supplementary Figure 1:

PARP1 poly(ADP-ribosyl)ates chromatin associated histones. (A) HEK293T cells were depleted of PARP1 and nuclei were prepared. The nuclei were incubated with 400 μM etheno-NAD^+ at 37°C for 20 min and lysed in SDS-lysis buffer. Western blotting was performed with the Ig4 antibody, which specifically recognizes the etheno-group of NAD^+. (B) HEK293T cells were transiently transfected with HA-PARP1 (ov. PARP1) or with an empty vector (control). Nuclei were prepared and incubated with 400 μM etheno-NAD^+ in presence or absence of the PARP-Inhibitor PJ34.

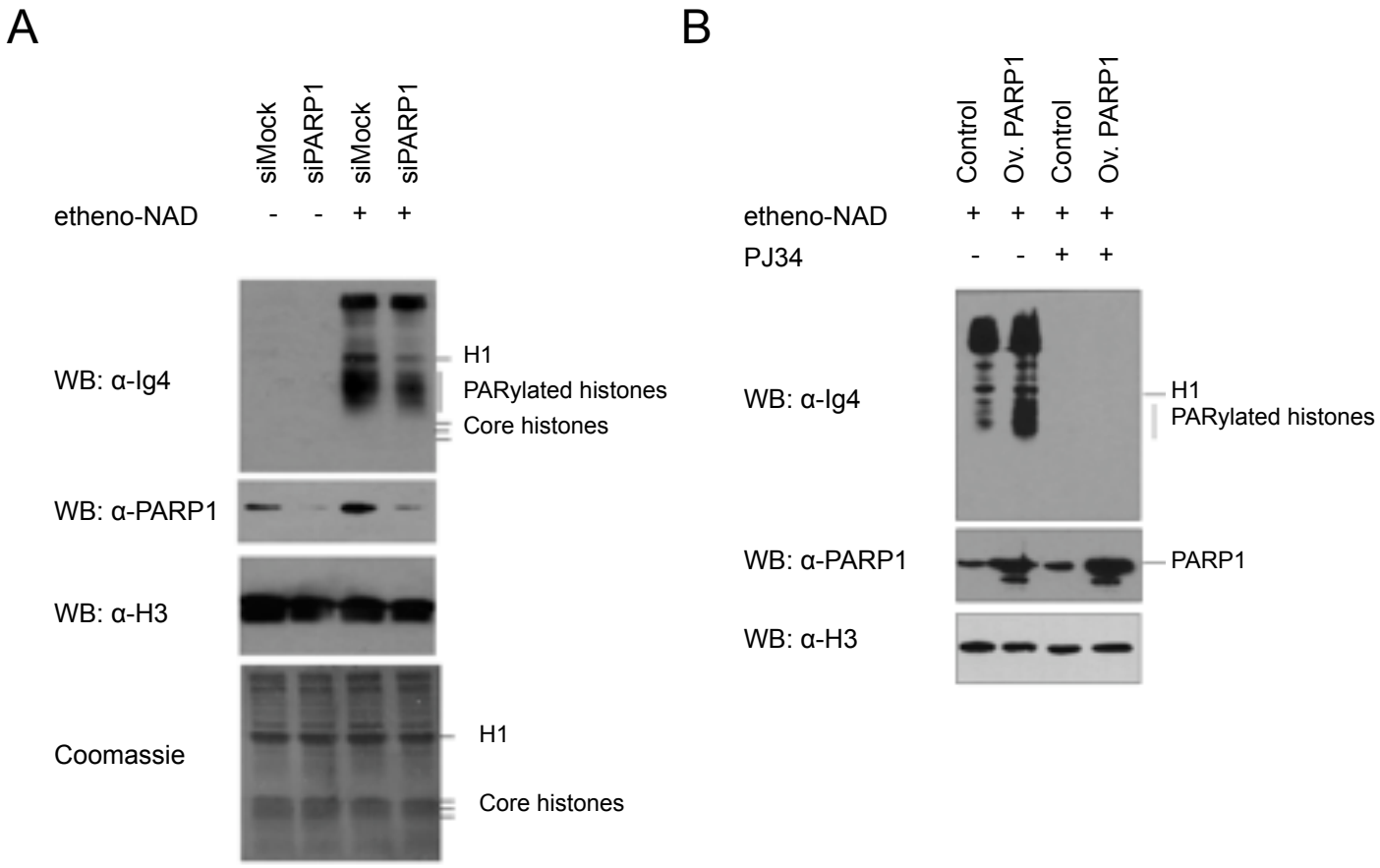
Supplementary Figure 2:

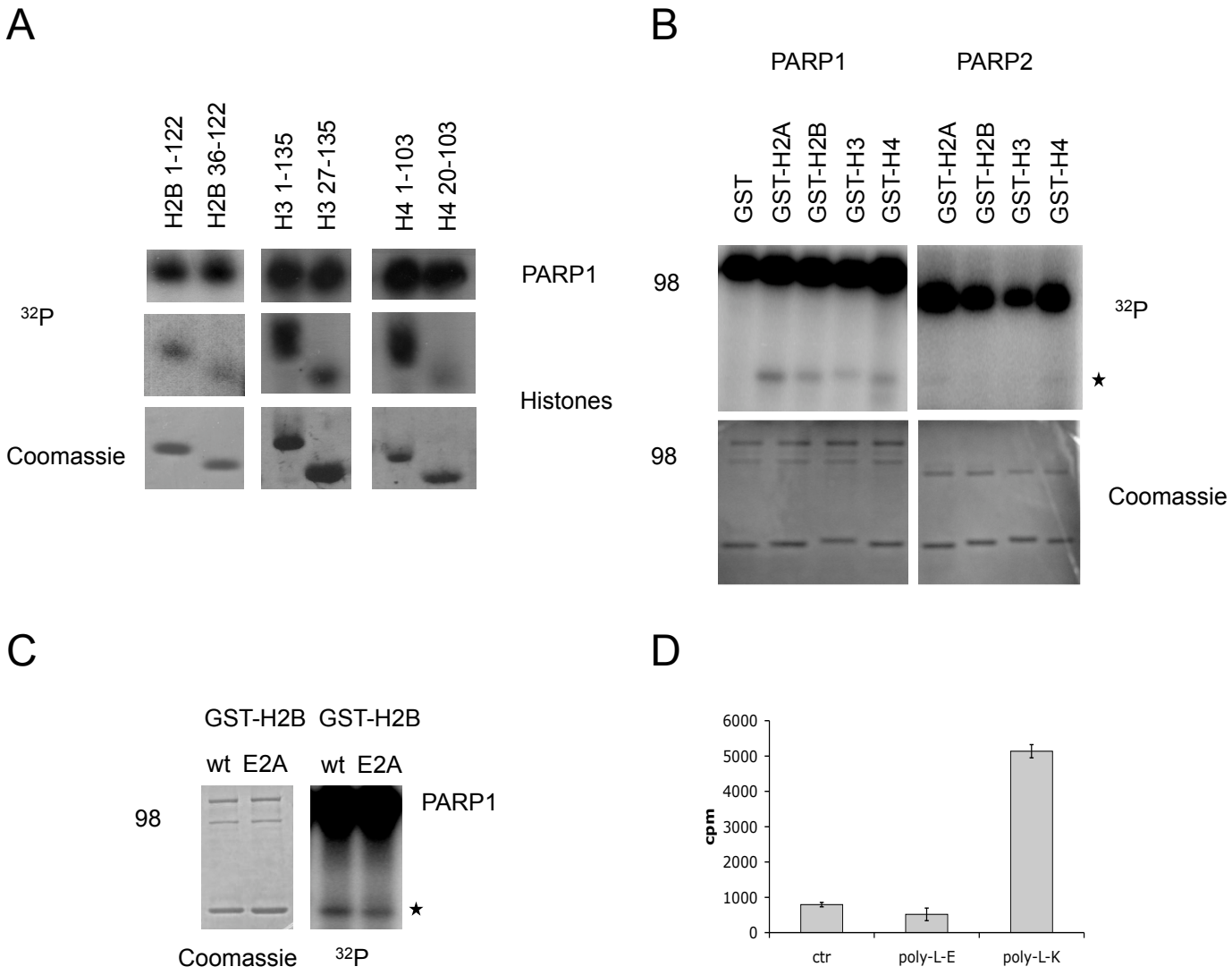
(A) Trans-ADP-ribosylation of histone tails by PARP1. 1.5 μg of full-length and truncated histones were incubated with 10 pmol PARP1 and 100 nM ^{32}P-NAD^+ for 15 min at 30°C. His-tagged H2B (36-122) was generated by PCR and cloned into pET3a with NdeI and BamHI restriction enzymes. The protein was expressed in inclusion bodies, solubilized, purified by a nickel-column and dialyzed against water. The other histones were expressed and purified as in Luger K. et al, 1997, JMB, 272, 301-311. (B) Identical to Fig. 1C of the main manuscript. The automodification of PARP2 was adjusted to the automodification of PARP1 by ImageQuant-Software. (C) Trans-ADP-ribosylation of H2B is not impaired in an H2B E2A mutant, in which the only glutamic acid residue is substituted by an alanine. Shown are autoradiographs and Coomassie stained gels. (D) Poly-L-lysine but not poly-L-glutamate are modified by hPARP1. Poly-L-amino acids were coupled onto cyanogen-bromide activated agarose beads over night as suggested by the provider (Sigma-Aldrich). Excess poly-L-amino acids were washed away and unoccupied reactive sites were blocked over night. The beads were washed and equilibrated in PARP1 reaction buffer. Reactions were performed for 5 minutes at 30°C in the presence of 100 nM radiolabeled NAD^+. The beads were washed 3 times in PARP1 reaction buffer containing 500 mM NaCl before scintillation counts in two different channels were determined. (E) Extracted ion chromatogram of the biotin tagged H3 (23-42) peptide, ADP-ribosylated by PARP1. ETD fragment spectrum of ADP-ribosylated

H3 peptide (590.29 m/z) at K37, indicated by the sequence plot. (F) Automodification of wild-type PARP1 and PARP1 D756K mutant for 10 min at 30°C in presence of activating DNA (EcoRI-linker) and 100 nM ^{32}P-NAD^{+}. Shown is an autoradiography and the coomassie stained gel. (G) Histone H4 (aa 1-22) peptide was ADP-ribosylated with PARP1 or PARP1 D756K mutant for 15 min at 30°C with 100 nM ^{32}P-NAD^{+}. The peptides were purified by microvolume-C18 reversed phase columns, eluted into scintillation liquid and counted for incorporated ^{32}P. Relative increase of counts per minutes was calculated over background (peptides added after termination of the reaction by 3AB) and the counts obtained for wild-type PARP1 were set to 100.

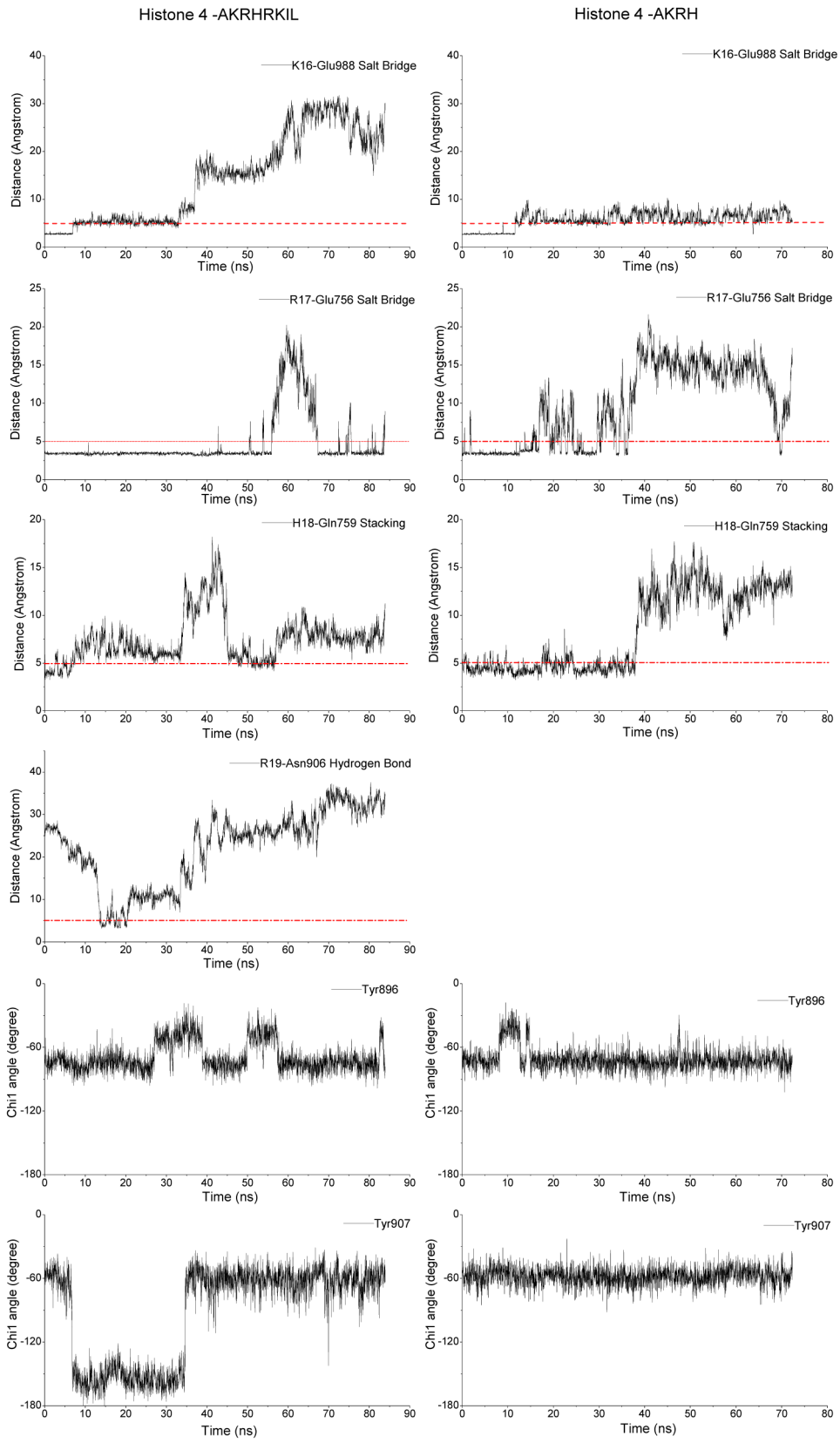
Supplementary Figure 3:

Time evolution of intermolecular salt bridges and side chain dihedral angles of PARP1 Tyrosine residues in the catalytic cleft.


A
siMock
siPARP1
siMock
siPARP1
etheno-NAD
- - + +
WB: α-Ig4
H1
PARylated histones
Core histones
WB: α-PARP1
WB: α-H3
Coomassie
H1
Core histones
B
Control
Ov. PARP1
Control
Ov. PARP1
etheno-NAD
+ + + +
PJ34
- - + +
WB: α-Ig4
H1
PARylated histones
WB: α-PARP1
PARP1
WB: α-H3


A
H2B 1-122
H2B 36-122
H3 1-135
H3 27-135
H4 1-103
H4 20-103
32P
PARP1
Histones
Coomassie
B
PARP1
PARP2
GST
GST-H2A
GST-H2B
GST-H3
GST-H4
GST-H2A
GST-H2B
GST-H3
GST-H4
98
98
32P
Coomassie
C
GST-H2B
GST-H2B
wt
E2A
wt
E2A
98
PARP1
Coomassie
32P
D
cpm
6000
5000
4000
3000
2000
1000
0
ctr
poly-L-E
poly-L-K
E
H3 K37-ADPR
Intensity
m/z
[M+5H]2+
[M+5H]3+
[M+5H]5+
Biotin-K-A-A-R-K-S-A-P-A-T-G-G-V-K-K*-P-H-R-Y-R
F
PARP1 wt
PARP1 D756K
32P
Coomassie
G
cpm
100
75
50
25
0
PARP1 wt
PARP1 D756K

Time evolution of intermolecular salt bridges and side chain dihedral angles of PARP1 Tyr residues in the catalytic cleft



Published online 16 April 2009 *Nucleic Acids Research, 2009, Vol. 37, No. 11* ***3723–3738***
doi:10.1093/nar/gkp229

Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites

Matthias Altmeyer[1,2], Simon Messner[1,2], Paul O. Hassa[3], Monika Fey[1] and Michael O. Hottiger[1,*]

[1]Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland, [2]Life Science Zurich Graduate School, Molecular Life Science Program, University of Zurich and [3]European Molecular Biology Laboratory (EMBL), Gene Expression Unit, Meyerhofstrasse 1, 69117 Heidelberg, Germany

Received January 14, 2009; Revised March 9, 2009; Accepted March 24, 2009

ABSTRACT

Poly(ADP-ribose) polymerase 1 (PARP1) synthesizes poly(ADP-ribose) (PAR) using nicotinamide adenine dinucleotide (NAD) as a substrate. Despite intensive research on the cellular functions of PARP1, the molecular mechanism of PAR formation has not been comprehensively understood. In this study, we elucidate the molecular mechanisms of poly(ADP-ribosyl)ation and identify PAR acceptor sites. Generation of different chimera proteins revealed that the amino-terminal domains of PARP1, PARP2 and PARP3 cooperate tightly with their corresponding catalytic domains. The DNA-dependent interaction between the amino-terminal DNA-binding domain and the catalytic domain of PARP1 increased V_{max} and decreased the K_m for NAD. Furthermore, we show that glutamic acid residues in the auto-modification domain of PARP1 are not required for PAR formation. Instead, we identify individual lysine residues as acceptor sites for ADP-ribosylation. Together, our findings provide novel mechanistic insights into PAR synthesis with significant relevance for the different biological functions of PARP family members.

INTRODUCTION

Poly(ADP-ribose) polymerases (PARPs) use nicotinamide adenine dinucleotide (NAD) as substrate to synthesize poly(ADP-ribose) (PAR) (1). On the cellular level, PAR formation has been implicated in a wide range of processes, such as maintenance of genomic stability, transcriptional regulation, energy metabolism and cell death (2).

PARP1 was the first protein described to catalyze PAR formation in response to mitogenic stimuli or genotoxic stress (3–7). It contains three functionally distinct domains: an amino-terminal DNA-binding domain (DBD), an auto-modification domain (AD) and a carboxyl-terminal PARP homology domain that includes the catalytic domain (CAT) responsible for PAR formation (8). The DBD extends from the initiator methionine to threonine 373 in human PARP1. It contains two structurally and functionally unique zinc fingers (FI: aa, amino acid, 11–89; FII: aa 115–199) (2,9). Recently, a third and so far unrecognized zinc-binding motif was discovered (FIII: aa 233–373) (10,11). The DBD also contains a bipartite nuclear localization signal (NLS) of the form KRK-X(11)-KKKSKK (aa 207–226) that targets PARP1 to the nucleus (12). The PARP1 zinc fingers FI and FII are thought to recognize altered structures in DNA rather than particular sequences and have also been reported to be involved in protein–protein interactions (13). PARP1 strongly associates with DNA single and double strand breaks generated either directly by DNA damage or indirectly by the enzymatic excision of damaged bases during DNA repair processes (2,9). Several studies indicate that the first zinc finger is required for PARP1 activation by both DNA single and double strand breaks, whereas the second zinc finger may exclusively act as a DNA single strand break sensor (2,9).

The AD of PARP1 is located in the central region of the enzyme, between residues 373 and 525 of human PARP1 (14,15). It was identified as the domain containing acceptor amino acids for the covalent attachment of PAR (16). In addition, several recent studies identified a weak leucine-zipper motif in the amino-terminal region of the AD, which suggests that this motif might be involved in homo- and/or hetero-dimerization (9). The AD of PARP1 also comprises a breast cancer 1 protein (BRCA1) C-terminus (BRCT) domain (from aa 386 to 464 in

*To whom correspondence should be addressed. Tel: +41 44 635 54 74; Fax: +41 44 635 68 40; Email: hottiger@vetbio.uzh.ch

Poly(ADP-Ribose) Polymerase 1 Promotes Tumor Cell Survival by Coactivating Hypoxia-Inducible Factor-1–Dependent Gene Expression

Michael Elser,[1] Lubor Borsig,[2] Paul O. Hassa,[4] Suheda Erener,[1] Simon Messner,[1] Taras Valovka,[1] Stephan Keller,[3] Max Gassmann,[3] and Michael O. Hottiger[1]

[1]Institute of Veterinary Biochemistry and Molecular Biology, [2]Zurich Center for Integrative Human Physiology (ZIHP) and Institute of Physiology, and [3]Zurich Center for Integrative Human Physiology (ZIHP) and Institute of Veterinary Physiology, University of Zurich, Zurich, Switzerland; and [4]Gene Expression Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

Abstract

Hypoxia-inducible factor 1 (HIF-1) is the key transcription factor regulating hypoxia-dependent gene expression. Lack of oxygen stabilizes HIF-1, which in turn modulates the gene expression pattern to adapt cells to the hypoxic environment. Activation of HIF-1 is also detected in most solid tumors and supports tumor growth through the expression of target genes that are involved in processes like cell proliferation, energy metabolism, and oxygen delivery. Poly(ADP-ribose) polymerase 1 (PARP1) is a chromatin-associated protein, which was shown to regulate transcription. Here we report that chronic myelogenous leukemia cells expressing small interfering RNA against PARP1, which were injected into wild-type mice expressing PARP1, showed tumor growth with increased levels of necrosis, limited vascularization, and reduced expression of GLUT-1. Of note, PARP1-deficient cells showed a reduced HIF-1 transcriptional activation that was dependent on PARP1 enzymatic activity. PARP1 neither influenced binding of HIF-1 to its hypoxic response element nor changed HIF-1α protein levels in hypoxic cells. However, PARP1 formed a complex with HIF-1α through direct protein interaction and coactivated HIF-1α–dependent gene expression. These findings provide convincing evidence that wild-type mice expressing PARP1 cannot compensate for the loss of PARP1 in tumor cells and strengthen the importance of the role of PARP1 as a transcriptional coactivator of HIF-1–dependent gene expression during tumor progression. (Mol Cancer Res 2008;6(2):282–90)

Received 8/10/07; revised 9/28/07; accepted 10/29/07.

Grant support: Swiss National Science Foundation grants 31-109315.05 and 31-112216 and the Kanton of Zurich (P.O. Hassa, M. Gassmann, and M.O. Hottiger).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Requests for reprints: Michael O. Hottiger, Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. Phone: 41-44-635-54-74; Fax: 41-44-635-68-40. E-mail: hottiger@vetbio.uzh.ch

doi:10.1158/1541-7786.MCR-07-0377

Introduction

In solid tumors, rapid cell proliferation is associated with areas of hypoxia. Intratumoral hypoxia induces neoangiogenesis, which is an essential switch from tumorigenesis to tumor progression (1). Oxygen limitation regulates vascularization, glucose metabolism, cell survival, and tumor spread. The hypoxic response critically depends on the transcription factor hypoxia-inducible factor-1 (HIF-1; ref. 2). HIF-1α was found to be overexpressed in more than 70% of human cancers and their metastases (3). The effect of HIF-1 on tumor growth is complex and involves the activation of several adaptive pathways and results in the induction of target genes (4). In solid tumors, immunohistochemistry often shows larger fronts of HIF nuclear expression delineating areas of necrosis (5). Induction of HIF is therefore believed to be supportive, if not causative, in cancer (6-8). In tumor xenograft and orthotopic mouse models, manipulation of the levels of either HIF-1α or HIF-2α has shown a causal link between HIF expression and tumor progression (4). HIF signaling has emerged as an important hypoxia-driven response allowing tumor cells to survive, expand, and invade. As a result, tumor hypoxia or HIF expression is strongly associated with a diminished therapeutic response and malignant progression (9).

HIF induction is a multistep process, which is tightly regulated *in vivo* (10, 11). HIF-1 is composed of two polypeptides: HIF-1α and HIF-1β (12). Two additional HIF-α members, the closely related HIF-2α (13) and more distantly related HIF-3α (14), were recently identified. HIF-1 activity is regulated at the posttranscriptional level by protein degradation of HIF-1α subunits after oxygen-dependent hydroxylation of specific proline residues (15). During hypoxia, the prolyl hydroxylases are inactive and HIF-1α is not complexed with the ubiquitin E3 ligase complex containing von Hippel Lindau, thereby allowing for the formation of active HIF-1 complexes (2, 5, 12). Transactivation involves dimerization of the two HIF-1 subunits, which bind to an enhancer element, called hypoxia response element, in target genes. Among the most studied promoters with regard to the recruitment of HIF-1 are those of the *EPO*, *GLUT-1*, and *CA9* [carbonic anhydrase IX (*CAIX*)] genes (16-18). The presence of hypoxia response element sites is necessary, but not sufficient, to direct gene expression in response to hypoxia, suggesting that HIF-1 must interact with other transcription factors or cofactors bound

Histone ADP-ribosylation revisited

Simon Messner[1,2] and Michael O. Hottiger[1]

[1]Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland, [2]Life Science Zurich Graduate School, Molecular Life Science Program

Running title: Histone ADP-ribosylation
Key words: ARTD1, PARP-1, NAD^+, ADP-ribosylation, histone, chromatin

Correspondence: Michael O. Hottiger, DVM, PhD, Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, email: hottiger@vetbio.uzh.ch

Abstract

Most of the published data on histone post-translational modifications focus on small covalent modifications such as acetylation, methylation and phosphorylation. *In vitro* ADP-ribosylation of histone proteins has been described as early as 1969 and was shortly after confirmed *in vivo*. Numerous laboratories subsequently reported histone ADP-ribosylation using purified cell nuclei, isolated nucleosomes or purified histones. ADP-ribosyltransferases belonging to two different families have been reported to ADP-ribosylate histones. The biological significance of histone ADP-ribosylation has been often restricted to functions attributed to the modifying enzymes. Until recently, the ADP-ribose acceptor sites of histones have not been identified due to the labile nature of the chemical linkage. However, recent structural and enzymological characterizations of ADP-ribosyltransferase family members, as well as the availability of genetically modified proteins and cells suggest that histone ADP-ribosylation plays a fundamental role in chromatin regulation. This review revisits the older literature and combines it with recent findings to provide an overview of histone ADP-ribosylation.

Protein ADP-ribosylation

ADP-ribosylation is a post-translational modification of proteins, catalyzed by ADP-ribosyltransferases that utilize NAD^+ as substrate [1]. ADP-ribosylation comprises the transfer of the ADP-ribose moiety from NAD^+ to specific amino acid residues on substrate proteins, also known as mono(ADP-ribosyl)ation. Subsequent elongation of the protein-bound ADP-ribose generates poly(ADP-ribosyl)ated proteins. Mono- and poly(ADP-ribosyl)ation of proteins are phylogenetically ancient, reversible post-translational modifications being implicated in a wide range of processes [2]. These include maintenance of genomic stability,

transcriptional regulation, energy metabolism and cell death, although in many instances the precise molecular consequences are not yet known.

Mono- versus poly(ADP-ribosyl)ation

In all eukaryotic tissues only low levels of protein-bound ADP-ribose residues are found. The total amount ranges from 5-60 pmols (about 3-30 ng) ADP-ribose per mg DNA [3,4]. Most ADP-ribose residues are bound to proteins as single ADP-ribose group rather than as oligomeric or polymeric ADP-ribose chains [5]. In mouse liver, the ratio is about 226:1 (mono- versus poly(ADP-ribosyl)ation), in human lymphocytes about 49:1 [6]. Comparison of chemically fractionated proteins of rat liver cells revealed that over 95% of mono(ADP-ribose) conjugates are located in other compartments than the nucleus (e.g. mitochondria) [7]. In contrast, most poly(ADP-ribose) chains are conjugated to nuclear proteins . The different subcellular distribution indicates independent functions of the ADP-ribose conjugate subclasses and suggests the involvement of at least two different enzyme classes, one able to catalyze mono(ADP-ribosyl)ation and the other additionally able to generate ADP-ribose polymers (see below).

Methods to measure ADP-ribosylation of histones

ADP-ribosylation (the attachment of ADP-ribose as monomer or polymer) of histones proteins has been investigated in the past by different *in vivo* and *in vitro* approaches. When radiolabeled ADP-ribose was administered intraperitoneally to rats, approximately one percent of the radioactivity was recovered in material of the liver nuclei 2 hours after injection [8]. Chromatography of this material revealed that the radioactivity co-eluted with the histone subfraction [8]. Since NAD^+ cannot permeate the cell membrane, no specific precursor is available for studies in intact tissues or in cell culture [9]. Labeling of histones in permeabilized mouse nuclei with radiolabeled NAD^+ predominantly generated mono(ADP-ribosyl)ated histones when a low concentration of radiolabeled NAD^+ (50μM) was employed [10]. At higher NAD^+ concentration (200μM) the ratio of poly- to mono(ADP-ribosyl)ated core histones increased [10], indicating that the cellular NAD^+ concentration is a critical factor for histone ADP-ribosylation. The generation of an antibody recognizing polymers of ADP-ribose (PAR) allowed later to confirm by immunoblot analysis that histones from whole cell extracts of asynchronized cells are indeed poly(ADP-ribosyl)ated *in vivo* [11]. Experiments with pure recombinant purified ADP-ribosyltransferases and different histone mutations allowed to

further elucidated histone ADP-ribosylation [12].

Histone ADP-ribosylation patterns

Histones isolated from rat liver nuclei and HeLa cells incubated with radiolabeled NAD^+ revealed that all histone proteins H1, H2A, H2B, H3 and H4 were modified by a copurified ADP-ribosyltransferase [13-15]. Also significant changes in the ADP-ribosylation pattern among histone variants of the same histone were observed [10]. The ADP-ribosylation of histones was not visible upon coomassie blue staining of SDS-polyacrylamide gels, but was only visualized by radiolabeled NAD^+, suggesting that the modified histones represent only a small fraction of total histones (less then 1%) [16-18]. Among the histone subfractions, H1 seemed to be the best acceptor, followed by H2B, while H2A, H3 and H4 were only weakly modified [19]. This order is almost comparable with those observed in isolated nuclei [15,20-22]. Efforts to exactly quantify the best acceptor turned out to be very difficult and depended on the different experimental protocols, cell types employed and different cultivation conditions [20,23-25]. Histones, especially nucleosomal core histones, were modified primarily by monomers or short oligomers rather than by long polymers [4,13,20,26-28]. A main chain length of 2-3 ADP-ribose units was found for histones H1 isolated from Ehrlich ascited tumor cell nuclei [29], whereas other labs have identified a chain length up to 15 ADP-ribose units attached to H1 [17,18,28]. In native chromatin, histone H1 was the major ADP-ribose acceptor, whereas in H1-depleted chromatin histone H2B became the major poly(ADP-ribose) histone acceptor protein [30], suggesting that ADP-ribosylation of histone proteins depend on the chromatin composition.

Two types of covalent chemical linkages are observed in ADP-ribosylated proteins

Radiolabeled ADP-ribsoylated histones were traditionally isolated from tissues and cells by precipitation with TCA. Extraction of modified proteins from the precipitated material by HCl revealed that both fractions (acid soluble and acid insoluble) contain ADP-ribosylated histones [8]. Part of the insoluble protein-bound radioactivity was rendered acid-soluble by pre-treatment with phosphodiesterase, a protein able to degrade polymers of ADP-ribose (see below). These observations indicate that while mono(ADP-ribosyl)ated proteins are likely acid soluble, poly(ADP-ribosyl)ated histones remain rather acid-insoluble [8]. Since complex formation was not observed when purified polymers of ADP-ribose were mixed with H1, the binding was suggested to be covalent [17]. Further treatment of acid-soluble and acid-insoluble (ADP-ribosyl)ated proteins with neutral hydroxylamine and alkali, resulted in the release of

free acid soluble ADP-ribose or 5'AMP respectively, indicating two types of bonds, both alkali-labile, but only one susceptible to neutral hydroxylamine [17,28,31]. Both types of bonds were equally distributed among acid-soluble and acid-insoluble (ADP-ribosyl)ated proteins [32,33]. Based on the sensitivity of ADP-ribose conjugates to neutral hydroxylamine (and to alkali), a larger portion of poly(ADP-ribosyl)ated histone bonds have been suggested to be carboxylesters [34]. Carboxylester bonds are predominantly found between two ADP-ribose units in the polymer of APD-ribose. Carboxylester bonds might potentially be found when the first ADP-ribose moiety would be covalently bound to an acidic amino acid, such as glutamic and aspartic acid residues [35] (Fig.1). The hydroxylamine-resistant but alkali-labile linkage was suggested to be a ketoamine bond [28,32,36]. This type of bond would only be formed if positively charged amino acids such as lysine or arginines serve as ADP-ribose acceptor sites (Fig.1). Interestingly, ADP-ribosylated proteins containing ADP-ribose conjugates belonging to this type of linkage were also described *in vivo* [37].

Non-covalent ADP-ribosylation of histones

While all histone modifications observed above are expected to be catalyzed by ADP-ribosyltransferases, also a covalent non-enzymatic ADP-ribosylation of histones was described earlier [36,38]. ADP-ribose is a potent histone glycation and glycoxidation agent *in vitro* [38]. Glycation is the covalent binding of an ADP-ribose molecule through Schiff base formation to lysine and/or arginine residues, which is stable upon treatment with hydroxylamine. Incubation of ADP-ribose with histones H1, H2A, H2B, and H4 *in vitro* at pH 7.5 and 37°C over night resulted in the formation of ketoamines derived from a Schiff base by an Amadori rearrangement [38-40]. Based on the long incubation time, which is substantially longer than common incubation times of ADP-ribosylation reactions, glycation needs only to be considered *in vivo* if an enzyme is able to generate high concentrations of ADP-ribose from NAD^+.

Enzymes described to ADP-ribosylate histone proteins

Currently, three families of proteins have been described to ADP-ribosylate proteins [1]. First, mammalian proteins with distant sequence homology to bacterial diphtheria toxin, called ARTDs (formerly known as PARPs [2,41]), second proteins with sequence homology to bacterial clostridium toxin, called ARTCs (formerly known as membrane associated ecto-ARTs) [42] and finally some proteins of the sirtuin family [2,43]. While ARTC family members are found on the cell membrane, some of the ARTDs and sirtuin family members are found in

the nucleus and were described to modify histones.

ARTD1 (PARP1):

The presence of a cellular enzyme that is able to synthesize poly(ADP-ribose) was already reported in the 1960ies [44-46]. Soon this activity was assigned to ARTD1. ARTD1 is associated with the chromatin of eukaryotic cells and localizes within internucleosomal linker regions of HeLa cell chromatin [47]. Poly(ADP-ribosyl)ation of histones includes first mono-modification of acceptor residues, subsequent elongation of the primary unit to a polymer, as well as branching of the polymer by α(1'-2')ribose-ribose linkage. All enzymatic reactions are carried out by ARTD1 [19,48]. The ADP-ribose-histone linkage synthesized by ARTD1 was mainly, but not completely labile in neutral hydroxylamine, but both alkali-labile, indicating the existence of both types of bonds (ester- and ketoamine bonds) [32,49,50]. Similar linkages were observed in crude chromatin or isolated nuclei [19,51]. Purified ARTD1 from calf thymus was able to modify all five individual histones *in vitro* [12,52-56]. When the concentration of DNA exceeds the amount of histones, the modification was lost [57-59]. The length of histone poly(ADP-ribosyl)ation was reported to change dependent on the NAD^+ concentrations [60]. Efficient activation of ARTD1 upon genotoxic stress is widely documented [2]. Two laboratories recently reported the activation of ARTD1 by post-translational modification or protein complex formation independent on DNA damage [61,62]. Interestingly, high H1 concentrations at low ionic strength and in absence of Mg^{2+} stimulated ARTD1 activity [56,59]. In the presence of histones, the K_m of ARTD1 for NAD^+ decreased from 80 μM to 25 μM and the V_{max} doubled [57].

Other ARTDs:

From the described ADP-ribosyltransferases only ARTD1, 2 and ARTD3 are described to be solely nuclear, whereas ARTD5 and 6 localize also in the cytoplasma [2]. Spermatocytes from ARTD2 (PARP2) knockout mice were described to contain hypoacetylated H4, although the functional contribution of ARTD2 is not yet elucidated [63]. Molecular and structural *in vitro* analysis recently revealed that ARTD2 is unable to modify free histones [12]. In a proteomic screen, ARTD3 (PARP3) was shown to interact with histones H2B and H3. Whether ARTD3 is able to modify histones is currently not clear [64]. ARTD10 (PARP10) localizes in cytoplasm and nucleus and is only able to mono(ADP-ribosyl)ate core histones [65-67]. The exact location of the other ARTD family members still needs to be investigated [67].

Sirtuins:

SIRT 4 and 6 of the sirtuins can mono-ADP-ribosylate histones as well [43,68,69]. The ADP-ribosylation activity of these sirtuins is, however, five orders of magnitude slower than their deacetylation activity, which questions the biological significance of this reaction [70].

Nuclear readers of ADP-ribose

Three non-covalent poly(ADP-ribose) binding modules were reported so far [71]. The first described binding motif contains of alternating hydrophobic and basic residues, which confers affinity for poly(ADP-ribose) [72]. This motif is found in several nuclear proteins, including histones. The non-covalent interaction is strong enough to evict histones *in vitro* from chromatin [73,74]. Evidence that this is also possible *in vivo* are currently missing. The second motif is called poly(ADP-ribose)-binding zinc finger (PBZ) and is found in a number of proteins involved in DNA-damage response an checkpoint activation, such as APLF (aprataxin PNK-like factor) and CHFR (checkpoint protein with FHA and RING domains) [75,76]. Finally, macrodomains were described to bind free and protein bound mono- and poly(ADP-ribose) [77-80]. Macrodomain are found in several ADP-ribosyltransferases, chromatin-remodeling enzymes (e.g. Alc1) and in the histone variants macroH2A1 and macroH2A2 [2,81-83]. Only the macrodomains of macroH2A1.1 and Alc1 were described to bind to the poly(ADP-ribosyl)ated form of PARP1 *in vivo* and to alter chromatin compaction, transiently [80,83].

ADP-ribosylation as transient modification

Histone ADP-ribosylation is a reversible modification, which contributes to the dynamic heterogeneous entities of nucleosomes. Two classes of enzymes are able to reverse ADP-ribosylation. ADP-ribosyl hydrolases (ARHs) and poly(ADP-ribose) glycohydrolases (PARGs) [42,84]. The importance of these enzyme in histone poly(ADP-ribosyl)ation metabolism is apparent from their key role in the rate-limiting step, i.e. the removal of primary ADP-ribosyl groups from acceptor proteins and in the overall turnover of poly(ADP-ribosyl) groups in the cell [85]. While ARH3 and PARGs are able to hydrolyze glycosidic bonds (between to ADP-ribose units or a glutamic acid residue and the primary ADP-ribose), they are not able to reverse ketoamine-linked ADP-ribose conjugates [86]. ARH3 and PARG act both as exo- and endoglycosidases [87]. An ADP-ribosyl protein lyase with molecular mass of 83kDA was once purified from rat liver and was able to remove the glutamic acid linked

ADP-ribosyl moieties [88,89]. Unfortunately, the gene for this protein was never cloned. While ARH2 is inactive, only ARH1 is able to specifically hydrolyze only arginine-ADP-ribose conjugates [90]. An enzyme able to hydrolyze lysine-ADP-ribose bonds has not yet been described. The absence of such an enzyme would leave a mono-ADP-ribose moiety at the protein, which would mark the protein irreversibly. Similarly, cleavage of poly(ADP-ribose) by a nuclear phosphodiesterase is predicted to leave a terminal phosphate group at the acceptor site, which cannot further be cleaved by PARG or extended by a ARTD.

Towards identifying the ADP-ribose acceptor sites in histones

It has been shown by trypsin digestion studies that the ADP-ribosylation of core histones predominantly takes place at the basic amino-terminal regions of the proteins [13,91]. Based on chemical properties of the linkages of the ADP-ribosylated tryptic fragmentation of the H1 and H2B, glutamic acid at position 2 of histone H2B [13] and of glutamic acid residues at position 2, 14 and 116 of H1, as well as to the carboxy-terminal COOH of lysine residue at position 213 of the same histone were suggested to be ADP-ribosylated [22,26,27]. The acceptor sites of other histone tails were never mapped, despite that they were modified [12,13,91]. Although the modification of glutamic acid residues as acceptor residues for histones was never verified using purified enzymes (e.g. ARTD1) or confirmed by mutagenesis or mass spectrometry, many review articles in the past three decades attributed the modification of glutamic acid residues to ARTD family members.

Recently the automodification sites of ARTD1 could be mapped and identified as lysine residues [50]. Furthermore, lysine residues in the tails of H3 and H4 were identified by mass spectrometry as ADP-ribose acceptor sites catalyzed by ARTD1, thus identifying lysine residues as acceptor sites for the ARTD family and resolving the paradox, that histones without glutamic acid residues at their amino-terminal tail can be ADP-ribosylated [12]. Since other ADP-ribosyltransferases (e.g. sirtuins) were described to modify only arginine residues, glutamic acid specific modifying enzymes remain to be identified [69,92-94]. Whether the modified glutamic acid residue is indeed catalyzed by an enzyme or induced non-enzymatically during the extraction procedure has to be further elucidated. Together, only enzymes able to modify lysine and arginine residues of histones were described so far.

Crosstalk of histone ADP-ribosylation with other post-translational modifications

Since both, lysine and arginine residues of histones were described to be modified by acetylation and methylation, respectively, a possible cross-talk between these two covalent

modifications of chromatin can be expected. Indeed, hyperacetylated chromatin regions associated with the chromatin domains that are undergoing poly(ADP-ribosylation), suggesting a crosstalk for lysine residues [95,96]. In proliferating cells or after stimulation of human lymphoid cells, the number of ADP-ribosyl groups equaled or exceeded by one the number of acetyl groups on histone H4 [10,97,98]. A 6 hour treatment with butyrate (a known histone deacetylase inhibitor) resulted in the loss of the correlation between acetylation and (ADP-ribosyl)ation, suggesting that acetylation might interfere with (ADP-ribosyl)ation and that the two modifications may share common functions [97]. Alternatively, H4 acetylation may constitute a signal for H4 ADP-ribosylation or *vice versa.* A crosstalk of histone arginine residues being ADP-ribosylated or methylated by protein arginine methyltransferase enzymes has not been described so far.

Histones modified by phosphorylation also appear to be poly(ADP-ribosyl)ated [96,99,100]. Using a reconstituted protein kinase assay system revealed that ADP-ribosylated histones are poor acceptors for the phosphorylation reaction [101]. In addition, phosphorylation of histones from calf thymus by cAMP-dependent protein kinase *in vitro* was markedly reduced when the histones were ADP ribosylated [102,103]. Although the majority of these experiments were performed *in vitro*, experiments on acetylation and ADP-riboslyation suggest that different cross talks very likely exist *in vivo*, possibly also involving other modification such as methylation or ubiquitinylation.

Several groups investigated the functional role of histone ADP-ribosylation during nuclear processes such as chromatin structure dynamics, replication, transcription or repair (Fig.2). While the influence of the histone modification is rather well established, little is know about the enzymes catalyzing the modification. The following sections mainly focuses articles investigating histone ADP-ribosylation also including the existing literature on ARTD1, while only little or none information is available about the other ARTD family members.

ADP-ribosylation-mediated nucleosome structure dynamics

Previous studies have shown that poly(ADP-ribose) is associated with the nucleosome [104,105]. ADP-ribosylation was reported to be involved in the regulation of the higher order structure of chromatin [106]. ARTD1 is the only so far described ARTD, which preferentially interacts with nucleosomes with a periodicity of 8-10 [107]. In absence of NAD^+ (unphysiological conditions) saturating binding of ARTD1 to nucleosomes promotes chromatin compaction as visualized by electron microscopy, whereas a high amount of NAD^+ induces ARTD1

automodification and its release from nucleosomes and subsequently chromatin relaxation *in vitro* [108-112]. Moreover, when these ADP-ribosylated polynucleosomes are in relaxed form, they cannot be condensed by increasing the ionic strength, showing a close similarity to H1 depleted chromatin *in vitro* [113]. Poly(ADP-ribosyl) groups on histones might thus be important at the replication fork [97]. Interestingly, modified H1 remained associated with chromatin *in vitro* [108,110]. The influence of poly(ADP-ribosyl)ation on the chromatin conformation was also studied *in vitro* by using monoclonal and polyclonal antibodies specific for individual histones. In poly(ADP-ribosyl)ated chromatin an increased accessibility of some histone tails to antibodies was observed [30]. The relaxed state of poly(ADP-ribosyl)ated polynucleosomes was also confirmed by sedimentation velocity analysis. When poly(ADP-ribose) is degraded by PARG activity, the chromatin returns to the condensed state [109]. Chromatin enriched in poly(ADP-ribosyl)ated nucleosomes appeared to be more nuclease sensitive than that found in the fraction of chromatin depleted of poly(ADP-ribosyl)ation [114]. In contrast to these studies, other *in vitro* studies suggested that poly(ADP-ribosyl)ation leads to a condensation of chromatin through the formation of covalent histone H1 poly(ADP-ribosyl)ation [11,17,18,115]. Whether the above described changes on the level of nucleosomes apply also *in vivo*, remains to be determined.

Histone ADP-ribosylation during the cell cycle and replication

Measurements of incorporated radiolabeled NAD^+ using permeabilized cells revealed that ADP-ribosylation takes place throughout the cell cycle [116]. Comparison of proliferating cells with contact-inhibited cells revealed that rates of poly(ADP-ribose) synthesis and DNA synthesis in permeabilized cells are highest during proliferation and especially in malignant cells and lowest in contact inhibited cells [117]. The idea that rapidly proliferating cells exhibit relatively higher activities of histone poly(ADP-ribosyl)ation than quiescent cells has been further supported by observation on SV-40 transformed cells, mitogen-stimulated lymphocytes and fertilizing sea urchin eggs [10,85,118-120]. Blocking cells by treatment with chemicals or serum depletion resulted in the synthesis of only mono- but not of poly(ADP-ribosyl)ated histones [97]. Characteristically, significantly more protein-bound poly(ADP-ribose) was also detectable in proliferating cells by immunoblot analysis using the 10H antibody generated against polymers of ADP-ribose [117]. Furthermore, an increase of poly(ADP-ribosyl)ation was observed during differentiation, suggesting that ADP-ribosylation might also be observed in specialized non-dividing cells [121,122].

In synchronized HeLa cells the amount of ADP-ribose polymers was found to increase from early S-phase to peak at the mid S-phase with a second, even larger increase seen at the S-G2 transition [14,98,116,123-127]. The importance of ADP-ribosylation was further strengthened by the observation that inhibition of ADP-ribosylation by 5-methylnicotinamide arrested the growth of HeLa cells between the end of S-phase or during mitosis [128]. Since not all cell lines are arrested by inhibition of ADP-ribosylation, a possible requirement of ADP-ribosylation for cell cycle progression needs to be further investigated. Recently the application of ADP-ribosylation inhibitors pursued into clinical trials phase II, displaying promising results in cancer therapy [129].

Histone ADP-ribosylation and transcription

Different analyses indicated that ADP-ribosylation activity was associated primarily with transcriptionally active regions, whereas the transcriptionally inert chromatin fractions were found to contain relatively low levels of ADP-ribosylation activity [14,130]. Recently, it was reported that histone H1 and ARTD1 exhibited a reciprocal pattern of chromatin binding at the promoters of actively transcribed genes [131]. Furthermore, ARTD1 could exclude H1 from a subset of ARTD1-stimulated promoters, suggesting a functional interplay between ARTD1 and H1 at the level of nucleosome binding [131]. Whether the enzymatic activity of ARTD1 is required for the observed effects remains to be defined. Several reports suggested a transcriptional coactivator role of ARTD1 independent of its enzymatic activity or not through the modification of histones [132-134]. ARTD1's enzymatic activity is important in puff formation in Drosophila at many loci, including Hsp70 [135]. Whether histones are ADP-ribosylated during puff formation was not elucidated. Through a RNAi screen of known coactivators, ARTD1 was recently identified as being necessary for the rapid loss of nucleosomes at the Hsp70 gene after heat shock [136]. Addition of an ADP-ribosylation inhibitor only 10 minutes before heat shock was sufficient to block the nucleosome eviction, indicating that ADP-ribosylation is required. It has been reported earlier that poly(ADP-ribosyl)ation of H3 and H4 results in their release from DNA [137,138]. Thus, nucleosomal displacement or even eviction may be regulated by histone ADP-ribosylation for the transcription of certain genes.

Histone ADP-ribosylation and DNA repair

Upon treatment of cells with alkylating agent (e.g. dimethyl sulfate), the levels of mono- and poly(ADP-ribosyl)ated proteins rose by a factor of 12 and 21 respectively [139]. The main non-

histone protein that becomes poly(ADP-ribosyl)ated in intact cells after DNA damage is ARTD1 itself [140]. The level of modification was found to involve only 4% of the total ARTD1 pool [21]. The principal poly(ADP-ribose) acceptor of alkylation induced ADP-riboslyation of histones were H2B and H3 [10,141]. Minor amounts of other histones, especially H4 and H1 were also modified under these conditions [139,141,142]. The preferred modification of histone H2B induced by DNA fragmentation may not be due to a marked acceptor specificity for a certain ADP-ribosyltransferase, but might more likely reflect an altered chromatin structure as caused by the DNA strand breaks where histone H1 is less accessible for poly(ADP-ribosyl)ation than histone H2B [32]. The chemistry and repairability of DNA breaks induced by different substances as well as the cell type may affect the pattern of mono and poly(ADP-ribosyl)ation.

Interestingly, induced ADP-ribosylation of histone H2B could not be removed with neutral hydroxylamine (but by treatment with alkali), indicating lysines and arginines as acceptor sites [32]. Similar experiments with ADP-ribosylated histones, isolated from the slime mold *physarum polycephalum,* suggested glutamate acceptor sites for H2A and H2B, and lysine, arginines acceptor sites for H3 and H4 [143].

Cleavage of the DNA with either DNase I or micrococcal nuclease to fragments of an average size of 10-20 kilobases dramatically induces the formation of poly(ADP-ribosyl)ated species of histones in nuclei [16]. Histones in a subset of nucleosomes proximal to the site of initial attack by micrococcal nuclease were found to be extensively ADP-ribosylated [14]. This interpretation has however, to be reconsidered, since later experiments suggested that the DNA strand breaks induced by micrococcal nuclease would activate ARTD1 and thus induce greater ADP-ribosylation of histone proteins [16].

Conclusions

Tremendous efforts have been undertaken over the last decades to decipher the physiological roles of ADP-ribosylation on the molecular level. ADP-ribosylation has not been included in the histone code, primarily because stimulation of ARTD1 activity, one of the best characterized nuclear ADP-ribosylating enzymes, and poly(ADP-ribosyl)ation of histones *in vivo* is best visible following the introduction of DNA strand breaks. Consequently, this has restricted the discussion of the biological significance to modification under genotoxic stress. In the light of the overwhelming amount of evidence supporting the functional role of histone ADP-ribosylation, we favor the view that this modification should be considered as a histone modification, taking place not only after DNA-damage, but also during other nuclear

processes, such as DNA-replication, transcription and chromatin remodeling, thus affecting chromatin function. Despite the development of genetic tools and the availability of new protocols for mass spectrometry, basic questions remain to be answered. Clearly, additional research will further improve our understanding of which enzymes are modifying distinct histone residues under a particular conditions and of the functions of histone ADP-ribosylation and their implications for several nuclear processes.

Acknowledgements

The authors acknowledge the contributions by researchers whose work could not be cited in this review due to space limitations. We thank M.Altmeyer and P.O.Hassa (both Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Zurich, Switzerland) for critical reading of the manuscript and helpful discussions. Our own research on ADP-ribosylation is supported by SNF grants 31-109315 and 31-122421 as well as by the Kanton of Zurich.

References

1 Hottiger, M. O., Hassa, P. O., Lüscher, B., schüler, H. & Koch-Nolte, F. Toward a unified nomenclature for mammalian ADP-ribosylatransferases. *Trends Biochem Sci* **accepted for publication** (2010).

2 Hassa, P. O., Haenni, S. S., Elser, M. & Hottiger, M. O. Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol Mol Biol Rev* **70**, 789-829 (2006).

3 Bredehorst, R., Wielckens, K., Adamietz, P., Steinhagen-Thiessen, E. & Hilz, H. Mono(ADP-ribosyl)ation and poly(ADP-ribosyl)ation of proteins in developing liver and in hepatomas: relation of conjugate subfractions to metabolic competence and proliferation rates. *Eur J Biochem* **120**, 267-274 (1981).

4 Minaga, T., Romaschin, A. D., Kirsten, E. & Kun, E. The in vivo distribution of immunoreactive larger than tetrameric polyadenosine diphosphoribose in histone and non-histone protein fractions of rat liver. *J Biol Chem* **254**, 9663-9668 (1979).

5 Wielckens, K., Bredehorst, R., Adamietz, P. & Hilz, H. Protein-bound polymeric and monomeric ADP-ribose residues in hepatic tissues. Comparative analyses using a new procedure for the quantification of poly(ADP-ribose). *Eur J Biochem* **117**, 69-74 (1981).

6 Wielckens, K., Garbrecht, M., Kittler, M. & Hilz, H. ADP-ribosylation of nuclear proteins in normal lymphocytes and in low-grade malignant non-Hodgkin lymphoma cells. *Eur J Biochem* **104**, 279-287 (1980).

7 Hilz, H. ADP-ribosylation of proteins--a multifunctional process. *Hoppe-Seyler's Z Physiol Chem* **362**, 1415-1425 (1981).

8 Ueda, K., Omachi, A., Kawaichi, M. & Hayaishi, O. Natural occurrence of poly(ADP-ribosyl) histones in rat liver. *Proc Natl Acad Sci USA* **72**, 205-209 (1975).

9 Nolde, S. & Hilz, H. Extracellular NAD as a cytostatic agent. *Hoppe-Seyler's Z Physiol Chem* **353**, 505-513 (1972).

10 Boulikas, T. At least 60 ADP-ribosylated variant histones are present in nuclei from dimethylsulfate-treated and untreated cells. *The EMBO Journal* **7**, 57-67 (1988).

11 Wong, M., Kanai, Y., Miwa, M., Bustin, M. & Smulson, M. Immunological evidence for the in vivo occurrence of a crosslinked complex of poly(ADP-ribosylated) histone H1. *Proc Natl Acad Sci USA* **80**, 205-209 (1983).

12 Messner, S. *et al.* PARP1 ADP-ribosylates lysine residues in core histone tails. *submitted* (2010).

13 Burzio, L. O., Riquelme, P. T. & Koide, S. S. ADP ribosylation of rat liver nucleosomal core histones. *J Biol Chem* **254**, 3029-3037 (1979).

14 Jump, D. B., Butt, T. R. & Smulson, M. Nuclear protein modification and chromatin substructure. 3. Relationship between poly(adenosine diphosphate) ribosylation and different functional forms of chromatin. *Biochemistry* **18**, 983-990 (1979).

15 Giri, C. P., West, M. H. & Smulson, M. Nuclear protein modification and chromatin substructure. 1. Differential poly(adenosine diphosphate) ribosylation of chromosomal proteins in nuclei versus isolated nucleosomes. *Biochemistry* **17**, 3495-3500 (1978).

16 Boulikas, T. DNA strand breaks alter histone ADP-ribosylation. *Proc Natl Acad Sci USA* **86**, 3499-3503 (1989).

17 Stone, P. R., Lorimer, W. S. & Kidwell, W. R. Properties of the complex between histone H1 and poly(ADP-ribose synthesised in HeLa cell nuclei. *Eur J Biochem* **81**, 9-18 (1977).

18 Nolan, N. L., Butt, T. R., Wong, M., Lambrianidou, A. & Smulson, M. E. Characterization of poly(ADP-ribose)--histone H1 complex formation in purified polynucleosomes and chromatin. *Eur J Biochem* **113**, 15-25 (1980).

19 Kawaichi, M., Ueda, K. & Hayaishi, O. Initiation of poly(ADP-ribosyl) histone synthesis by poly(ADP-ribose) synthetase. *J Biol Chem* **255**, 816-819 (1980).

20 Okayama, H., Ueda, K. & Hayaishi, O. Purification of ADP-ribosylated nuclear proteins by covalent chromatography on dihydroxyboryl polyacrylamide beads and their characterization. *Proc Natl Acad Sci USA* **75**, 1111-1115 (1978).

21 Krupitza, G. & Cerutti, P. Poly(ADP-ribosylation) of histones in intact human keratinocytes. *Biochemistry* **28**, 4054-4060 (1989).

22 Riquelme, P. T., Burzio, L. O. & Koide, S. S. ADP ribosylation of rat liver lysine-rich histone in vitro. *J Biol Chem* **254**, 3018-3028 (1979).
23 Tanuma, S. I., Enomoto, T. & Yamada, M. A. Distribution of poly(ADP-ribose) in histones of HeLa cell nuclei. *Biochem Biophys Res Commun* **74**, 599-605 (1977).
24 Rickwood, D., MacGillivray, A. J. & Whish, W. J. The modification of nuclear proteins by ADP-ribosylation. *Eur J Biochem* **79**, 589-598 (1977).
25 Mennella, M. R., Quesada, P., Farina, B., Leone, E. & Jones, R. ADP-ribosylation of nuclear proteins in mouse testis. *Biochem J* **205**, 245-248 (1982).
26 Ogata, N., Ueda, K. & Hayaishi, O. ADP-ribosylation of histone H2B. Identification of glutamic acid residue 2 as the modification site. *J Biol Chem* **255**, 7610-7615 (1980).
27 Ogata, N., Ueda, K., Kagamiyama, H. & Hayaishi, O. ADP-ribosylation of histone H1. Identification of glutamic acid residues 2, 14, and the COOH-terminal lysine residue as modification sites. *J Biol Chem* **255**, 7616-7620 (1980).
28 Adamietz, P., Bredehorst, R. & Hilz, H. ADP-ribosylated histone H1 from HeLa cultures. Fundamental differences to (ADP-ribose)n-histone H1 conjugates formed into vitro. *Eur J Biochem* **91**, 317-326 (1978).
29 Braeuer, H. C., Adamietz, P., Nellessen, U. & Hilz, H. ADP-ribosylated histone H1. Isolation from Ehrlich-ascites-tumor-cell nuclei and partial characterization. *Eur J Biochem* **114**, 63-68 (1981).
30 Huletsky, A. *et al.* The effect of poly(ADP-ribosyl)ation on native and H1-depleted chromatin. A role of poly(ADP-ribosyl)ation on core nucleosome structure. *J Biol Chem* **264**, 8878-8886 (1989).
31 Goebel, M., Stone, P. R., Lengyel, H. & Hilz, H. Conversion of ADP-ribose to 5'-AMP by alkaline treatment and its use for an optical quantitation of mono and poly(ADP-ribose) residues in the micromolar range. *Hoppe-Seyler's Z Physiol Chem* **358**, 13-21 (1977).
32 Adamietz, P. & Hilz, H. Poly(adenosine diphosphate ribose) is covalently linked to nuclear proteins by two types of bonds. *Hoppe-Seyler's Z Physiol Chem* **357**, 527-534 (1976).
33 Hilz, H. & Stone, P. Poly(ADP-ribose) and ADP-ribosylation of proteins. *Rev Physiol Biochem Pharmacol* **76**, 1-58, 177 (1976).
34 Nishizuka, Y. *et al.* Enzymic adenosine diphosphoribosylation of nuclear proteins. *Cold Spring Harb Symp Quant Biol* **34**, 781-786 (1969).
35 Hilz, H., Adamietz, P., Bredehorst, R. & Wielckens, K. ADP-ribosylation of nuclear proteins. *Adv Enzyme Regul* **17**, 195-211 (1978).
36 Kun, E., Chang, A. C., Sharma, M. L., Ferro, A. M. & Nitecki, D. Covalent modification of proteins by metabolites of NAD+. *Proc Natl Acad Sci USA* **73**, 3131-3135 (1976).
37 Payne, D. M., Jacobson, E. L., Moss, J. & Jacobson, M. K. Modification of proteins by mono(ADP-ribosylation) in vivo. *Biochemistry* **24**, 7540-7549 (1985).
38 Cervantes-Laurean, D., Jacobson, E. L. & Jacobson, M. K. Glycation and glycoxidation of histones by ADP-ribose. *J Biol Chem* **271**, 10461-10469 (1996).
39 Cervantes-Laurean, D., Minter, D. E., Jacobson, E. L. & Jacobson, M. K. Protein glycation by ADP-ribose: studies of model conjugates. *Biochemistry* **32**, 1528-1534 (1993).
40 Cervantes-Laurean, D., Loflin, P. T., Minter, D. E., Jacobson, E. L. & Jacobson, M. K. Protein modification by ADP-ribose via acid-labile linkages. *J Biol Chem* **270**, 7929-7936 (1995).
41 Hassa, P. O. & Hottiger, M. O. The diverse biological roles of mammalian PARPS, a small but powerful family of poly-ADP-ribose polymerases. *Front Biosci* **13**, 3046-3082 (2008).
42 Koch-Nolte, F., Kernstock, S., Mueller-Dieckmann, C., Weiss, M. S. & Haag, F. Mammalian ADP-ribosyltransferases and ADP-ribosylhydrolases. *Front Biosci* **13**, 6716-6729 (2008).
43 Haigis, M. C. & Sinclair, D. A. Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol* **5**, 253-295, doi:10.1146/annurev.pathol.4.110807.092250 (2010).
44 CHAMBON, P., WEILL, J. D. & Mandel, P. Nicotinamide mononucleotide activation of new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. *Biochem Biophys Res Commun* **11**, 39-43 (1963).
45 Hasegawa, S., Fujimura, S., Shimizu, Y. & Sugimura, R. The polymerization of adenosine 5'-diphosphate-ribose moiety of NAD by nuclear enzyme. II. Properties of the reaction product. *Biochim Biophys Acta* **149**, 369-376 (1967).

46 Reeder, R. H., Ueda, K., Honjo, T., Nishizuka, Y. & Hayaishi, O. Studies on the polymer of adenosine diphosphate ribose. II. Characterization of the polymer. *J Biol Chem* **242**, 3172-3179 (1967).
47 Giri, C. P., West, M. H., Ramirez, M. L. & Smulson, M. Nuclear protein modification and chromatin substructure. 2. Internucleosomal localization of poly(adenosine diphosphate-ribose) polymerase. *Biochemistry* **17**, 3501-3504 (1978).
48 Kawaichi, M., Ueda, K. & Hayaishi, O. Multiple autopoly(ADP-ribosyl)ation of rat liver poly(ADP-ribose) synthetase. Mode of modification and properties of automodified synthetase. *J Biol Chem* **256**, 9483-9489 (1981).
49 Moss, J., Yost, D. A. & Stanley, S. J. Amino acid-specific ADP-ribosylation. *J Biol Chem* **258**, 6466-6470 (1983).
50 Altmeyer, M., Messner, S., Hassa, P. O., Fey, M. & Hottiger, M. O. Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites. *Nucleic Acids Research* **37**, 3723-3738, doi:10.1093/nar/gkp229 (2009).
51 Ueda, K., Kawaichi, M., Okayama, H. & Hayaishi, O. Poly(ADP-ribosy)ation of nuclear proteins. Enzymatic elongation of chemically synthesized ADP-ribose-histone adducts. *J Biol Chem* **254**, 679-687 (1979).
52 Okazaki, H., Niedergang, C. & Mandel, P. Adenosine diphosphate ribosylation of histone H1 by purified calf thymus polyadenosine diphosphate ribose polymerase. *Biochimie* **62**, 147-157 (1980).
53 Okazaki, H. *et al.* In vitro ADP-ribosylation of histones by purified calf thymus polyadenosine diphosphate ribose polymerase. *FEBS Lett* **110**, 227-229 (1980).
54 Poirier, G. G., Niedergang, C., Champagne, M., Mazen, A. & Mandel, P. Adenosine diphosphate ribosylation of chicken-erythrocyte histones H1, H5 and high-mobility-group proteins by purified calf-thymus poly(adenosinediphosphate-ribose) polymerase. *Eur J Biochem* **127**, 437-442 (1982).
55 Caplan, A. I., Niedergang, C., Okazaki, H. & Mandel, P. Poly ADP-ribose polymerase: self-ADP-ribosylation, the stimulation by DNA, and the effects on nucleosome formation and stability. *Arch Biochem Biophys* **198**, 60-69 (1979).
56 Ferro, A. M. & Olivera, B. M. Poly(ADP-ribosylation) in vitro. Reaction parameters and enzyme mechanism. *J Biol Chem* **257**, 7808-7813 (1982).
57 Okayama, H., Edson, C. M., Fukushima, M., Ueda, K. & Hayaishi, O. Purification and properties of poly(adenosine diphosphate ribose) synthetase. *J Biol Chem* **252**, 7000-7005 (1977).
58 Yoshihara, K., Hashida, T., Yoshihara, H., Tanaka, Y. & Ohgushi, H. Enzyme-bound early product of purified poly(ADP-ribose) polymerase. *Biochem Biophys Res Commun* **78**, 1281-1288 (1977).
59 Tanaka, Y., Hashida, T., Yoshihara, H. & Yoshihara, K. Bovine thymus poly(ADP-ribose) polymerase histone-dependent and Mg2+-dependent reaction. *J Biol Chem* **254**, 12433-12438 (1979).
60 Huletsky, A. *et al.* Sequential ADP-ribosylation pattern of nucleosomal histones. ADP-ribosylation of nucleosomal histones. *Eur J Biochem* **146**, 277-285 (1985).
61 Kauppinen, T. M. *et al.* Direct phosphorylation and regulation of poly(ADP-ribose) polymerase-1 by extracellular signal-regulated kinases 1/2. *Proc Natl Acad Sci USA* **103**, 7136-7141, doi:10.1073/pnas.0508606103 (2006).
62 Cohen-Armon, M. *et al.* DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation. *Molecular Cell* **25**, 297-308, doi:10.1016/j.molcel.2006.12.012 (2007).
63 Dantzer, F. *et al.* Poly(ADP-ribose) polymerase-2 contributes to the fidelity of male meiosis I and spermiogenesis. *Proc Natl Acad Sci USA* **103**, 14854-14859, doi:10.1073/pnas.0604252103 (2006).
64 Ewing, R. M. *et al.* Large-scale mapping of human protein-protein interactions by mass spectrometry. *Mol Syst Biol* **3**, 89, doi:10.1038/msb4100134 (2007).

65 Chou, H.-Y. E., Chou, H. T. & Lee, S.-C. CDK-dependent activation of poly(ADP-ribose) polymerase member 10 (PARP10). *J Biol Chem* **281**, 15201-15207, doi:10.1074/jbc.M506745200 (2006).
66 Yu, M. *et al.* PARP-10, a novel Myc-interacting protein with poly(ADP-ribose) polymerase activity, inhibits transformation. *Oncogene* **24**, 1982-1993, doi:10.1038/sj.onc.1208410 (2005).
67 Kleine, H. *et al.* Substrate-assisted catalysis by PARP10 limits its activity to mono-ADP-ribosylation. *Molecular Cell* **32**, 57-69, doi:10.1016/j.molcel.2008.08.009 (2008).
68 Hawse, W. F. & Wolberger, C. Structure-based mechanism of ADP ribosylation by sirtuins. *Journal of Biological Chemistry*, 1-25, doi:10.1074/jbc.M109.024521 (2009).
69 Fahie, K. *et al.* Side chain specificity of ADP-ribosylation by a sirtuin. *FEBS J* **276**, 7159-7176, doi:10.1111/j.1742-4658.2009.07427.x (2009).
70 Kowieski, T. M., Lee, S. & Denu, J. M. Acetylation-dependent ADP-ribosylation by Trypanosoma brucei Sir2. *J Biol Chem* **283**, 5317-5326, doi:10.1074/jbc.M707613200 (2008).
71 Kleine, H. & Lüscher, B. Learning how to read ADP-ribosylation. *Cell* **139**, 17-19, doi:10.1016/j.cell.2009.09.018 (2009).
72 Pleschke, J. M., Kleczkowska, H. E., Strohm, M. & Althaus, F. R. Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. *J Biol Chem* **275**, 40974-40980, doi:10.1074/jbc.M006520200 (2000).
73 Panzeter, P. L. *et al.* Targeting of histone tails by poly(ADP-ribose). *J Biol Chem* **268**, 17662-17664 (1993).
74 Althaus, F. R. *et al.* Histone shuttling by poly ADP-ribosylation. *Mol Cell Biochem* **138**, 53-59 (1994).
75 Ahel, I. *et al.* Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins. *Nature* **451**, 81-85, doi:10.1038/nature06420 (2008).
76 Isogai, S. *et al.* Solution structure of a zinc-finger domain that binds to poly-ADP-ribose. *Genes to cells : devoted to molecular & cellular mechanisms*, doi:10.1111/j.1365-2443.2009.01369.x (2010).
77 Karras, G. I. *et al.* The macro domain is an ADP-ribose binding module. *The EMBO Journal* **24**, 1911-1920, doi:10.1038/sj.emboj.7600664 (2005).
78 Kustatscher, G., Hothorn, M., Pugieux, C., Scheffzek, K. & Ladurner, A. G. Splicing regulates NAD metabolite binding to histone macroH2A. *Nat Struct Mol Biol* **12**, 624-625, doi:10.1038/nsmb956 (2005).
79 Dani, N. *et al.* Combining affinity purification by ADP-ribose-binding macro domains with mass spectrometry to define the mammalian ADP-ribosyl proteome. *Proc Natl Acad Sci USA* **106**, 4243-4248, doi:10.1073/pnas.0900066106 (2009).
80 Timinszky, G. *et al.* A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation. *Nat Struct Mol Biol*, doi:10.1038/nsmb.1664 (2009).
81 Pehrson, J. R. & Fried, V. A. MacroH2A, a core histone containing a large nonhistone region. *Science* **257**, 1398-1400 (1992).
82 Chakravarthy, S. *et al.* Structural characterization of the histone variant macroH2A. *Molecular and Cellular Biology* **25**, 7616-7624, doi:10.1128/MCB.25.17.7616-7624.2005 (2005).
83 Gottschalk, A. *et al.* Poly(ADP-ribosyl)ation directs recruitment and activation of an ATP-dependent chromatin remodeler. *Proc Natl Acad Sci USA*, doi:10.1073/pnas.0906920106 (2009).
84 Moss, J., Zolkiewska, A. & Okazaki, I. ADP-ribosylarginine hydrolases and ADP-ribosyltransferases. Partners in ADP-ribosylation cycles. *Adv Exp Med Biol* **419**, 25-33 (1997).
85 Ueda, K. & Hayaishi, O. ADP-ribosylation. *Annu Rev Biochem* **54**, 73-100, doi:10.1146/annurev.bi.54.070185.000445 (1985).
86 Oka, S., Kato, J. & Moss, J. Identification and characterization of a mammalian 39-kDa poly(ADP-ribose) glycohydrolase. *J Biol Chem* **281**, 705-713, doi:10.1074/jbc.M510290200 (2006).
87 D'Amours, D., Desnoyers, S., D'Silva, I. & Poirier, G. G. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J* **342 (Pt 2)**, 249-268 (1999).

88 Okayama, H., Honda, M. & Hayaishi, O. Novel enzyme from rat liver that cleaves an ADP-ribosyl histone linkage. *Proc Natl Acad Sci USA* **75**, 2254-2257 (1978).
89 Oka, J., Ueda, K., Hayaishi, O., Komura, H. & Nakanishi, K. ADP-ribosyl protein lyase. Purification, properties, and identification of the product. *J Biol Chem* **259**, 986-995 (1984).
90 Takada, T., Iida, K. & Moss, J. Cloning and site-directed mutagenesis of human ADP-ribosylarginine hydrolase. *J Biol Chem* **268**, 17837-17843 (1993).
91 Jump, D. B., Sudhakar, S., Tew, K. D. & Smulson, M. Probes to study the effect of methyl nitrosourea on ADP-ribosylation and chromatin structure at the subunit level. *Chem Biol Interact* **30**, 35-51 (1980).
92 Moss, J. & Vaughan, M. Isolation of an avian erythrocyte protein possessing ADP-ribosyltransferase activity and capable of activating adenylate cyclase. *Proc Natl Acad Sci USA* **75**, 3621-3624 (1978).
93 Moss, J. & Stanley, S. J. Amino acid-specific ADP-ribosylation. Identification of an arginine-dependent ADP-ribosyltransferase in rat liver. *J Biol Chem* **256**, 7830-7833 (1981).
94 Yost, D. A. & Moss, J. Amino acid-specific ADP-ribosylation. Evidence for two distinct NAD:arginine ADP-ribosyltransferases in turkey erythrocytes. *J Biol Chem* **258**, 4926-4929 (1983).
95 Malik, N. & Smulson, M. A relationship between nuclear poly(adenosine diphosphate ribosylation) and acetylation posttranslational modifications. 1. Nucleosome studies. *Biochemistry* **23**, 3721-3725 (1984).
96 Wong, M. & Smulson, M. A relationship between nuclear poly(adenosine diphosphate ribosylation) and acetylation posttranslational modifications. 2. Histone studies. *Biochemistry* **23**, 3726-3730 (1984).
97 Boulikas, T. Poly(ADP-ribosylated) histones in chromatin replication. *J Biol Chem* **265**, 14638-14647 (1990).
98 Boulikas, T., Bastin, B., Boulikas, P. & Dupuis, G. Increase in histone poly (ADP-ribosylation) in mitogen-activated lymphoid cells. *Exp Cell Res* **187**, 77-84 (1990).
99 Wong, M., Miwa, M., Sugimura, T. & Smulson, M. Relationship between histone H1 poly(adenosine diphosphate ribosylation) and histone H1 phosphorylation using anti-poly(adenosine diphosphate ribose) antibody. *Biochemistry* **22**, 2384-2389 (1983).
100 Tikoo, K., Lau, S. S. & Monks, T. J. Histone H3 phosphorylation is coupled to poly-(ADP-ribosylation) during reactive oxygen species-induced cell death in renal proximal tubular epithelial cells. *Mol Pharmacol* **60**, 394-402 (2001).
101 Tanigawa, Y., Tsuchiya, M., Imai, Y. & Shimoyama, M. Mono (ADP-ribosyl)ation of hen liver nuclear proteins suppresses phosphorylation. *Biochem Biophys Res Commun* **113**, 135-141 (1983).
102 Tanigawa, Y., Tsuchiya, M., Imai, Y. & Shimoyama, M. ADP-ribosylation regulates the phosphorylation of histones by the catalytic subunit of cyclic AMP-dependent protein kinase. *FEBS Lett* **160**, 217-220 (1983).
103 Ushiroyama, T. *et al.* Amino acid sequence of histone H1 at the ADP-ribose-accepting site and ADP-ribose X histone-H1 adduct as an inhibitor of cyclic-AMP-dependent phosphorylation. *Eur J Biochem* **151**, 173-177 (1985).
104 Aubin, R. J., Dam, V. T., Miclette, J., Brousseau, Y. & Poirier, G. G. Chromosomal protein poly(ADP-ribosyl)ation in pancreatic nucleosomes. *Can J Biochem* **60**, 295-305 (1982).
105 Aubin, R. J. *et al.* Hyper(ADP-ribosyl)ation of histone H1. *Can J Biochem* **60**, 1085-1094 (1982).
106 K Janakidevi, J. C. S. Print Service. *Novel AD-Ribosylations of Regularory Enzymaes and Proteins*, 111-119 (1980).
107 Butt, T. R., Jump, D. B. & Smulson, M. E. Nucleosome periodicity in HeLa cell chromatin as probed by micrococcal nuclease. *Proc Natl Acad Sci USA* **76**, 1628-1632 (1979).
108 Poirier, G. G., de Murcia, G., Jongstra-Bilen, J., Niedergang, C. & Mandel, P. Poly(ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure. *Proc Natl Acad Sci USA* **79**, 3423-3427 (1982).
109 de Murcia, G. *et al.* Modulation of chromatin superstructure induced by poly(ADP-ribose) synthesis and degradation. *J Biol Chem* **261**, 7011-7017 (1986).

110 Aubin, R. J. *et al.* Correlation between endogenous nucleosomal hyper(ADP-ribosyl)ation of histone H1 and the induction of chromatin relaxation. *The EMBO Journal* **2**, 1685-1693 (1983).
111 Kim, M. Y., Mauro, S., Gévry, N., Lis, J. T. & Kraus, W. L. NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. *Cell* **119**, 803-814, doi:10.1016/j.cell.2004.11.002 (2004).
112 Wacker, D. A. *et al.* The DNA binding and catalytic domains of poly(ADP-ribose) polymerase 1 cooperate in the regulation of chromatin structure and transcription. *Molecular and Cellular Biology* **27**, 7475-7485, doi:10.1128/MCB.01314-07 (2007).
113 Thoma, F., Koller, T. & Klug, A. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J Cell Biol* **83**, 403-427 (1979).
114 Hough, C. J. & Smulson, M. E. Association of poly(adenosine diphosphate ribosylated) nucleosomes with transcriptionally active and inactive regions of chromatin. *Biochemistry* **23**, 5016-5023 (1984).
115 Butt, T. R., DeCoste, B., Jump, D. B., Nolan, N. & Smulson, M. Characterization of a putative poly(adenosine diphosphate ribose)-chromatin complex. *Biochemistry* **19**, 5243-5249 (1980).
116 Adolph, K. W. Cell cycle variations in ADP-ribosylation of HeLa nuclear proteins. *Arch Biochem Biophys* **243**, 427-438 (1985).
117 Kun, E., Kirsten, E., Bauer, P. I. & Ordahl, C. P. Quantitative correlation between cellular proliferation and nuclear poly (ADP-ribose) polymerase (PARP-1). *Int J Mol Med* **17**, 293-300 (2006).
118 Lehmann, A. R., Kirk-Bell, S., Shall, S. & Whish, W. J. The relationship between cell growth, macromolecular synthesis and poly ADP-ribose polymerase in lymphoid cells. *Exp Cell Res* **83**, 63-72 (1974).
119 Berger, N. A., Adams, J. W., Sikorski, G. W., Petzold, S. J. & Shearer, W. T. Synthesis of DNA and poly(adenosine diphosphate ribose) in normal and chronic lymphocytic leukemia lymphocytes. *J Clin Invest* **62**, 111-118, doi:10.1172/JCI109094 (1978).
120 Rochette-Egly, C., Ittel, M. E., Bilen, J. & Mandel, P. Effect of nicotinamide on RNA and DNA synthesis and on poly(ADP-ribose) polymerase activity in normal and phytohemagglutinin stimulated human lymphocytes. *FEBS Lett* **120**, 7-11 (1980).
121 Chabert, M. G., Niedergang, C. P., Hog, F., Partisani, M. & Mandel, P. Poly(ADPR)polymerase expression and activity during proliferation and differentiation of rat astrocyte and neuronal cultures. *Biochim Biophys Acta* **1136**, 196-202 (1992).
122 Meyer-Ficca, M. L., Scherthan, H., Bürkle, A. & Meyer, R. G. Poly(ADP-ribosyl)ation during chromatin remodeling steps in rat spermiogenesis. *Chromosoma* **114**, 67-74, doi:10.1007/s00412-005-0344-6 (2005).
123 Colyer, R. A., Burdette, K. E. & Kidwell, W. R. Poly ADP-ribose synthesis and DNA replication in synchronized mouse L-cells. *Biochem Biophys Res Commun* **53**, 960-966 (1973).
124 Miwa, M., Sugimura, T., Inui, N. & Takayama, S. Poly(adenosine diphosphate ribose) synthesis during the cell cycle of transformed hamster lung cells. *Cancer Res* **33**, 1306-1309 (1973).
125 Kidwell, W. R. & Mage, M. G. Changes in poly(adenosine diphosphate-ribose) and poly(adenosine diphosphate-ribose) polymerase in synchronous HeLa cells. *Biochemistry* **15**, 1213-1217 (1976).
126 Wielckens, K., George, E., Pless, T. & Hilz, H. Stimulation of poly(ADP-ribosyl)ation during Ehrlich ascites tumor cell "starvation" and suppression of concomitant DNA fragmentation by benzamide. *J Biol Chem* **258**, 4098-4104 (1983).
127 Singh, N. & Cerutti, P. Poly ADP-ribosylation of histones in tumor promoter phorbol-12-myristate-13-acetate treated mouse embryo fibroblasts C3H10T1/2. *Biochem Biophys Res Commun* **132**, 811-819 (1985).
128 Kidwell, W. R. & Burdette, K. E. Poly(ADP-ribose) synthesis and cell division. *Biochem Biophys Res Commun* **61**, 766-774 (1974).

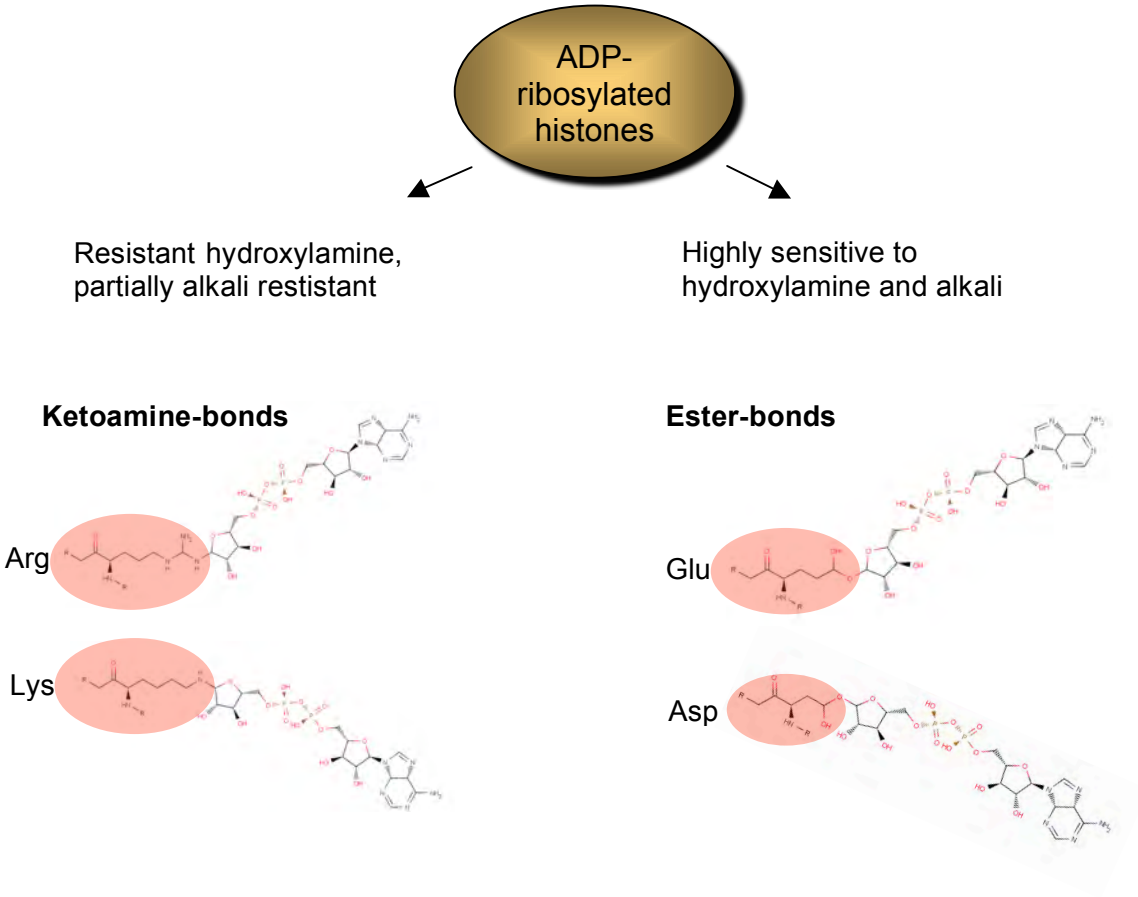
129 Papeo, G. *et al.* Poly(ADP-ribose) polymerase inhibition in cancer therapy: are we close to maturity? *Expert Opin Ther Pat* **19**, 1377-1400, doi:10.1517/13543770903215883 (2009).
130 Mullins, D. W., Giri, C. P. & Smulson, M. Poly(adenosine diphosphate-ribose) polymerase: the distribution of a chromosome-associated enzyme within the chromatin substructure. *Biochemistry* **16**, 506-513 (1977).
131 Krishnakumar, R. *et al.* Reciprocal binding of PARP-1 and histone H1 at promoters specifies transcriptional outcomes. *Science* **319**, 819-821, doi:10.1126/science.1149250 (2008).
132 Hassa, P. O., Covic, M., Hasan, S., Imhof, R. & Hottiger, M. O. The enzymatic and DNA binding activity of PARP-1 are not required for NF-kappa B coactivator function. *J Biol Chem* **276**, 45588-45597, doi:10.1074/jbc.M106528200 (2001).
133 Pavri, R. *et al.* PARP-1 determines specificity in a retinoid signaling pathway via direct modulation of mediator. *Molecular Cell* **18**, 83-96, doi:10.1016/j.molcel.2005.02.034 (2005).
134 Ju, B.-G. *et al.* Activating the PARP-1 sensor component of the groucho/ TLE1 corepressor complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway. *Cell* **119**, 815-829, doi:10.1016/j.cell.2004.11.017 (2004).
135 Tulin, A. & Spradling, A. Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at Drosophila puff loci. *Science* **299**, 560-562, doi:10.1126/science.1078764 (2003).
136 Petesch, S. J. & Lis, J. T. Rapid, transcription-independent loss of nucleosomes over a large chromatin domain at Hsp70 loci. *Cell* **134**, 74-84, doi:10.1016/j.cell.2008.05.029 (2008).
137 Wesierska-Gadek, J. & Sauermann, G. The effect of poly(ADP-ribose) on interactions of DNA with histones H1, H3 and H4. *Eur J Biochem* **173**, 675-679 (1988).
138 Benezra, R., Cantor, C. R. & Axel, R. Nucleosomes are phased along the mouse beta-major globin gene in erythroid and nonerythroid cells. *Cell* **44**, 697-704 (1986).
139 Adamietz, P. & Rudolph, A. ADP-ribosylation of nuclear proteins in vivo. Identification of histone H2B as a major acceptor for mono- and poly(ADP-ribose) in dimethyl sulfate-treated hepatoma AH 7974 cells. *J Biol Chem* **259**, 6841-6846 (1984).
140 Adamietz, P. Poly(ADP-ribose) synthase is the major endogenous nonhistone acceptor for poly(ADP-ribose) in alkylated rat hepatoma cells. *Eur J Biochem* **169**, 365-372 (1987).
141 Kreimeyer, A., Wielckens, K., Adamietz, P. & Hilz, H. DNA repair-associated ADP-ribosylation in vivo. Modification of histone H1 differs from that of the principal acceptor proteins. *J Biol Chem* **259**, 890-896 (1984).
142 Surowy, C. S. & Berger, N. A. Unique acceptors for poly(ADP-ribose) in resting, proliferating and DNA-damaged human lymphocytes. *Biochim Biophys Acta* **740**, 8-18 (1983).
143 Golderer, G. & Gröbner, P. ADP-ribosylation of core histones and their acetylated subspecies. *Biochem J* **277 (Pt 3)**, 607-610 (1991).

Figure legends:

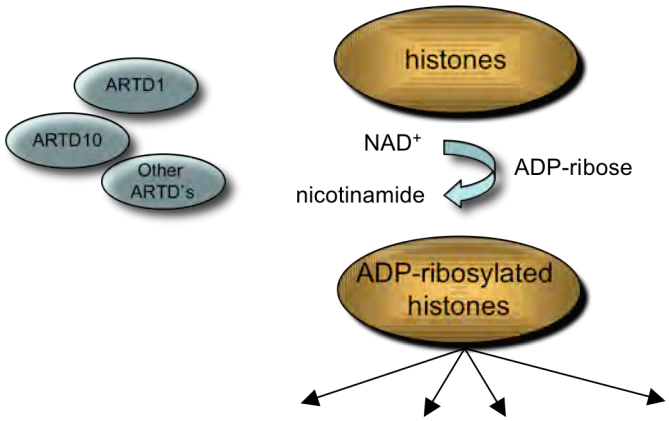
Figure 1: Two types of chemical linkages between proteins and ADP-ribose. Ketoamine bonds of Ω-N-(C1-ADP-ribosyl)-L-arginine and ε-N-(C1-ADP-ribosyl)-L-lysine are resistant to treatment with neutral hydroxylamine and partially alkali resistant. Carboxylester bonds of ε-O-(C1-ADP-ribosyl)-L-glutamate and δ-O-(C-1-ADP-ribosyl)-L-aspartate are highly sensitive to neutral hydroxylamine and alkali.

Figure 2: Involvement of ADP-ribosylated histones in cellular processes. ADP-ribosyltransferases catalyze the hydrolysis of NAD^+ into ADP-ribose and nicotinamide. ARTD1, ARTD10 and possibly by other ARTD family members modify histone proteins. ADP-ribosylated histones are involved in regulation of the chromatin structure, regulation of the cell cylce and are targeted during DNA-repair and transcription.

Messner and Hottiger (Figure 1)



Messner and Hottiger (Figure 2)



	Chromatin structure	Cell cycle	Transcription	DNA-repair
In vitro	•Chromatin relaxation [108-112] •Chromatin condensation [11,17,18,115]		•Release from H3 and H4 [137,138]	•H2B and H3 modification [10,141]
In vivo		•S-phase [14,98,116,123-127] •Proliferation [117] •Differentiation [121, 122]	•Active regions [14,130] •H1 release [131] •Nucleosome eviction [136]	•Chromatin compaction [80]

3.2 Unpublished Results

3.2.1 HDAC4 enhances PARP1 sumoylation

Several SUMO E3 ligases were reported to enhance SUMO modification of substrate proteins (159). Since we were interested in proteins that enhance PARP1 sumoylation, several E3 ligases were tested. Although PIAS proteins enhanced PARP1 sumoylation *in vitro* (unpublished results from Dr. Stefan Müller), overexpression of PIAS1-4, as well as TOPORS overexpression did not enhance PARP1 sumoylation (data not shown). Recently, also class IIa HDACs were shown to facilitate SUMO modification of several proteins (100-102). We tested therefore HDAC4 as a putative SUMO E3 ligase for PARP1 sumoylation (Fig. 4). Overexpression of Flag-HDAC4 with HA-PARP1 and myc-SUMO3 in HEK293T cells markedly increased the SUMO modification of PARP1 (Fig. 4A).

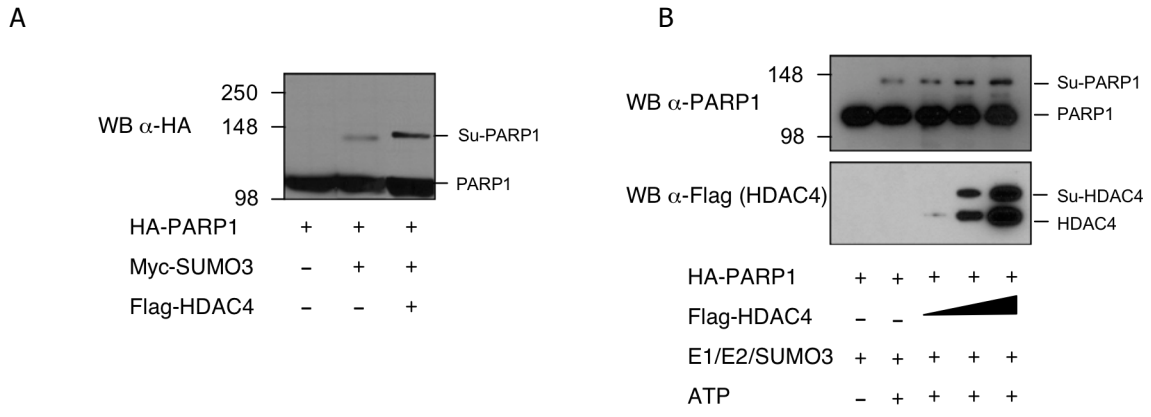


Figure 4: PARP1 sumoylation is enhanced in the presence of HDAC4 *in vivo* and *in vitro*. (A) HEK293T cells were transiently transfected with HA-PARP1, myc-SUMO3 and/or Flag-HDAC4. Whole cell extracts were taken 36 hours after transfection and analyzed by western blot with anti-HA antibody. The upper band at approximately 140 kDa corresponds to sumoylated PARP1. (B) Flag-HDAC4 was expressed by a baculovirus expression system in insect cells and purified with nickel beads. A standard *in vitro* sumoylation reaction was performed in the presence of HA-PARP1 and increasing amounts of Flag-HDAC4 (0.1μg, 0.5μg, 2.0μg Flag-HDAC4). The reaction proceeded for 30 min at 30°C. Samples were subjected to western blot with the indicated antibodies.

To further validate, if this observation was due to a direct interaction of HDAC4 with PARP1, we incubated purified PARP1 with increasing amounts of purified HDAC4 together with SUMO3 in a standard *in vitro* sumoylation assay. Indeed, the addition of HDAC4 increased sumoylation of PARP1 (Fig. 4B), suggesting that HDAC4 acts as a SUMO E3 ligase *in vitro* and *in vivo* (Fig. 4). Consistent with previous reports, HDAC4 was SUMO-modified as well (160).

3.2.2 HDAC4 does not directly deacetylate PARP1

Since HDAC4 complexes were described to deacetylate proteins, we further tested, whether HDAC4 could deacetylate PARP1. PARP1 was acetylated *in vitro* by p300 and acetyl-CoA. The reaction (± acetyl-CoA) was split and incubated with different amounts of purified HDAC4. After 1 hour incubation, acetylation of PARP1 was analyzed by western blot, using the E4 antibody, which recognizes acetylated K498, 505 and K508 of PARP1. The presence of HDAC4 alone did not decrease the acetylated levels of PARP1 (Fig. 5A). To investigate whether cellular HDAC4 associated complexes could deacetylate PARP1, overexpressed Flag-HDAC4 was immunoprecipitated from HEK293T cells and subsequently incubated with p300-acetylated PARP1 and assessed for acetylated levels of PARP1 as described above (Fig. 5B).

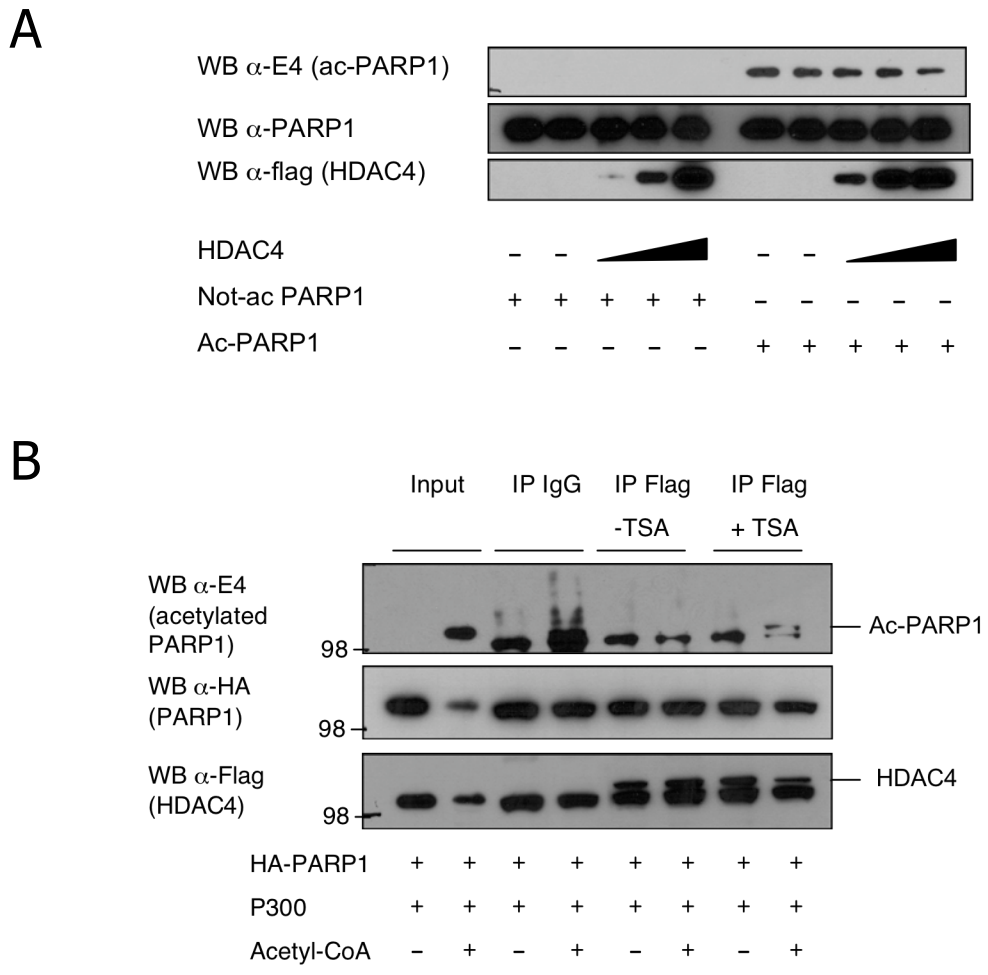


Figure 5: (A) PARP1 is not deacetylated by purified HDCAC4. Purified PARP1 was acetylated by p300 *in vitro*, without (not-ac PARP1) or in the presence of acetyl-CoA (ac PARP1). Equal amounts of the reactions were incubated with increasing amounts of purified Flag-HDAC4 (0.1 μg, 1.0 μg, 2.0 μg) for 2 hours at 30°C. The

samples were analyzed by western blot and probed with the indicated antibodies. (B) PARP1 is deacetylated by immunoprecipitated HDAC4 complex. Purified PARP1 was acetylated with p300 in vitro. Equal amounts of PARP1 were incubated with 10 μg of immunoprecipitated nuclear extract from HEK293T, transfected with Flag-HDAC4. IP was performed with antibody against IgG or Flag-Epitope. HDAC-inhibitor TSA (1μM) was added to the indicated samples. The reaction was incubated for 1 hour at 30°C.

Interestingly, immunoprecipitated HDAC4 was able to deacetylate PARP1. Addition of the HDAC-inhibitor trichostatin A (TSA) inhibited this activity (Fig. 5B). No deacetylation activity was observed for the IgG control reaction, suggesting that immunoprecipitated HDAC4 or HDAC4-associated proteins are able to deacetylate PARP1.

3.2.3 Members of the Sir2 family deacetylate PARP1

The Sir2 family of proteins consist of seven members (SIRT1-7). These proteins utilize NAD^+ as co-factor to cleave the acetyl-group from modified lysines, leading to the generation of O-acetyl-ADP-ribose and nicotinamide. Since PARP1 and SIRTs use the same co-factor, an interplay between these enzymes is very likely. To test whether SIRTs could deacetylate PARP1, purified GST-tagged SIRT1, a SIRT1 catalytic inactive mutant, SIRT2, SIRT6 and SIRT7 were incubated with a catalytic inactive PARP1 fragment (aa 373-1014), which was beforehand acetylated by p300 (Fig. 6).

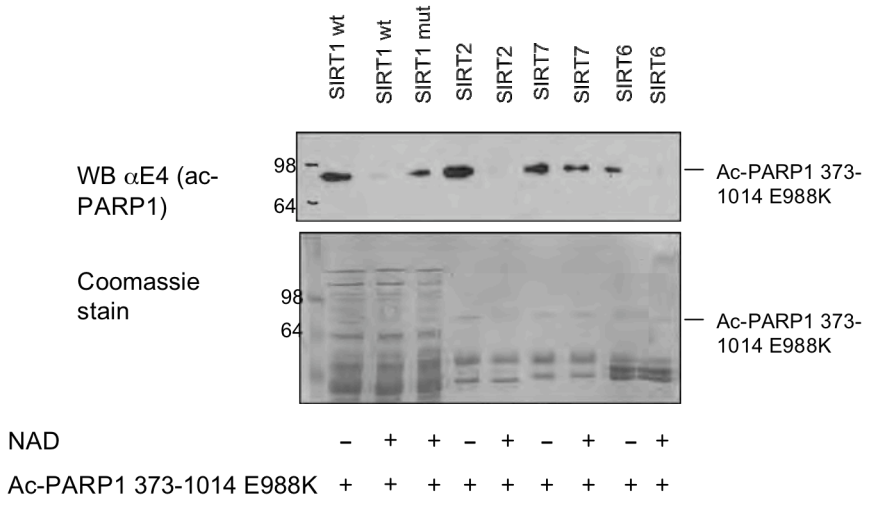


Figure 6: PARP1 is deacetylated by SIRT1, SIRT2 and SIRT6 in a NAD^+-dependent manner. Purified PARP1 373-1014 E988K was acetylated by p300 *in vitro*. The acetylation reaction was split and incubated with 1μg purified GST-SIRT1, GST-SIRT1 catalytic mutant, SIRT2, SIRT6 or SIRT7 for 1 hour at 30°C. The reaction contained acetylated PARP1 fragment 373-1014 and 1mM NAD, where indicated. Samples were analyzed by western blot for acetylated PARP1 with anti-E4 antibody, which recognizes acetylated PARP1.

SIRT1, 2 and SIRT6, but not SIRT7, were able to deacetylate the acetylated PARP1 fragment (Fig. 6). Deacetylation of PARP1 was NAD^+-dependent, suggesting that the

enzymatic activity of SIRTs was responsible for the observed effect. Indeed, a catalytic inactive mutant of SIRT1 was not longer able to deacetylate PARP1. Whether PARP1 can be deacetylated by sirtuins *in vivo*, remains to be investigated.

3.2.4 PCAF acetylates the zinc-finger I fragment of PARP1

Previously, it was observed that the histone acetyltransferase PCAF is able to acetylate PARP1 between amino acids 1-214 and 372-524, but the exact acetylation sites were not identified (161). In order to determine putative acceptor sites of PCAF-mediated PARP1 acetylation, several lysine mutations in the context of the 1-112 fragment of PARP1 were analyzed in an acetylation assay (Fig. 7/8). Only lysines were mutated, which would be structurally accessible for acetylation. As positive control, wild-type PARP1 fragment 1-112 was acetylated by PCAF (Fig. 7/8). Acetylation was furthermore observed for the K86/87/97R mutant fragment, but not for the K97/105/108R fragment and the K5R fragment, where K86/87/97/105/108 was mutated (Fig. 7/8). These results suggest that K105 and K108 are putative acceptor sites for PCAF mediated PARP1 acetylation. Whether K97 is also modified, remains to be investigated by a single PARP1 amino acid mutant or by other techniques, such as mass spectrometry.

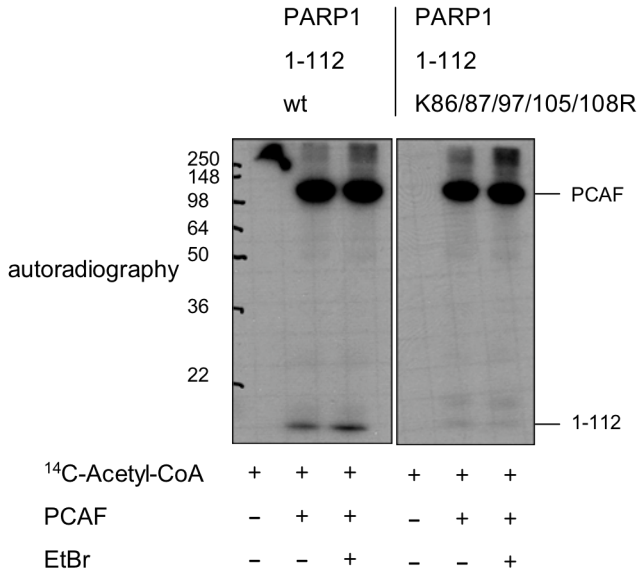


Figure 7: PARP1 is acetylated by PCAF in the region 1-112. Purified PARP1 fragments 1-112 were incubated for 1 hour at 30°C with purified PCAF, ^{14}C-acetyl-CoA and ethidiumbromide. Samples were resolved on SDS-PAGE and incorporated ^{14}C-acetyl-CoA was visualized by autoradiography.

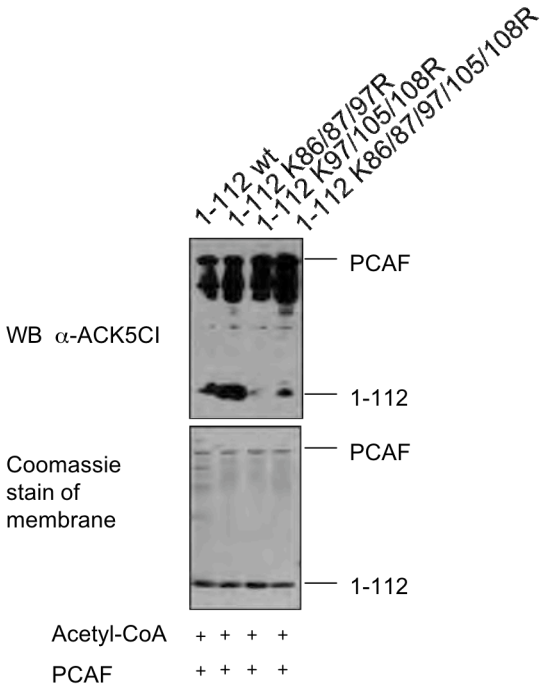


Figure 8: PARP1 is potentially acetylated by PCAF at lysines 97, 105, 108 within the PARP1 1-112 fragment. 1 g PCAF was incubated with mutated PARP1 1-112 fragments and acetyl-CoA for 1 hour at 30°C. The samples were probed by western blot with the general acetyl-recognizing antibody ACK5CI.

3.2.5 PARP2 is sumoylated by SUMO3

Within the PARP superfamily, PARP2 shares the highest sequence homology with PARP1. Therefore, we tested whether PARP2, comparable to PARP1, would be sumoylated *in vitro*. Purified PARP2 from insect cells was tested in an *in vitro* standard sumoylation assay including SUMO3 (Fig. 9).

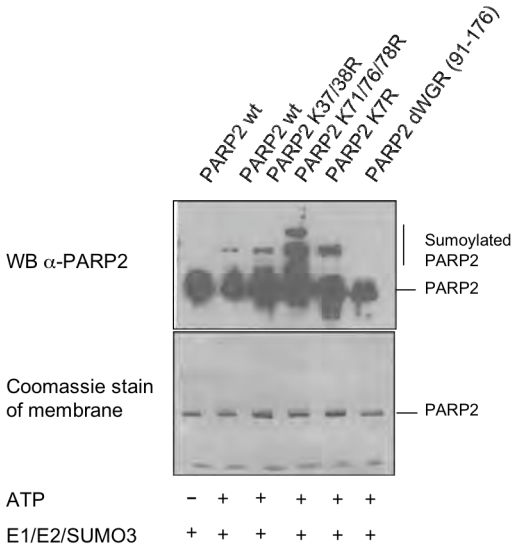


Figure 9: PARP2 is sumoylated by SUMO3 *in vitro*. Purified human PARP2 proteins were incubated in a standard sumoylation reaction with SUMO3 for 30 min at 30°C. Samples were resolved on SDS-PAGE and analyzed by a PARP2 antibody. PARP2 K7R: K37/38/48/49/56/59/71R.

PARP2 was sumoylated *in vitro* by SUMO3 (Fig. 9). Surprisingly, sumoylation was even enhanced when several lysines were mutated (K37/38/71/76/78R). Interestingly, sumoylation of PARP2 was completely lost, if the WGR domain was deleted (Fig. 9). Within this domain, only one SUMO-consensus motif around K143 is found. Thus, K143 could be a putative acceptor site of SUMO in PARP2. Further experiments including single amino acid substitution (e.g. K143R) have to be performed, to confirm this observation.

3.2.6 Material and Methods

Reagents

The radio-labelled ^{14}C-acetyl-CoA was purchased from Movarek Biochemicals and Radiochemicals. Antibodies against recombinant PARP1 and PARP2 were generated in our laboratory. The antibody against HA-tag was purchased from Covance, Flag (M2) antibody was from Sigma, ACK5CI was from Santa Cruz, anti acetyl-PARP1 E4 was generated together with the Monoclonal antibody core facility at the EMBL Monterotondo.

Generation and purification of recombinant proteins

Recombinant his-tagged PARP1, PARP2 proteins as well as all histone acetyltransferases were generated in Sf21 cells using the BacPAK (Clontech) or the pQE (Qiagen) system. Purification was performed in batch using Ni^{2+}-beads/Nitrobond (Invitrogen). Recombinant SUMO E1 activating enzyme was expressed in BL21(DE3) cells and purified via Glutathione-Sepharose (GE Healthcare) and Ni^{2+}-beads/Nitrobond (Invitrogen). SUMO conjugating enzyme Ubc9 and SUMO3 was also expressed in BL21(DE3) and purified using Ni^{2+}-beads/Nitrobond (Invitrogen).

In vitro sumoylation assay

The reaction was carried out in standard SUMO reaction buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 10% Glycerol, 0.5 mM DTT). 5 mM ATP was added to start the reaction. The incubation time was 30 min at 30°C, unless otherwise indicated. The final concentration of proteins was 100 nM for SAE1/SAE2, 500 nM Ubc9, 5 μM SUMO1/SUMO3 and 500 nM PARP2.

HAT-Assay

0.5 μg of PARP1 fragments were acetylated *in vitro* by recombinant p300 (1 μg) or PCAF (1 μg) as described elsewhere (162).

Deacetylation assay by sirtuins

Acetylated PARP1 was incubated in the same buffer as for acetylation, including 1 μg recombinant sirtuins and 1 mM NAD^+ in total volume of 30 μl for 1 hour at 30°C.

Immunoprecipitation of Flag-HDAC4

Immunoprecipitation of nuclear extracts was performed with Flag-antibody (M2) using IP-binding buffer (20 mM HEPES, 150 mM NaCl, 0.25% NP-40, 1 μg/ml Protease inhibitors). The salt concentration was increased to 50 mM KCl for washing steps. Beads were subsequently washed with HAT-buffer and *in vitro* acetylated PARP1 was incubated with the beads. Deacetylation reaction by HDAC4 was performed for 1 hour at 30°C.

4. Discussion and Perspectives

4.1 Summary of results

The articles described in this thesis established a regulatory role for sumoylation of PARP1 in regard to its transcriptional co-activator function, as well as the discovery of lysine ADP-ribosylation in the auto-modification domain of PARP1 and in histone tails.

In the first article, sumoylation was identified as a novel posttranslational modification of PARP1. We reported the modification of PARP1 by SUMO1 and SUMO3 (163). Lysine 486, within the auto-modification domain, was found to be the main sumoylated residue of PARP1. Neither the DNA-binding activity nor the enzymatic activity of PARP1 was altered by sumoylation. Instead, the acetylation of adjacent lysine residues by p300 was impaired of SUMO-modified PARP1. Additionally, we observed an increased acetylation status of the SUMO-deficient PARP1 mutant and a higher transcriptional co-activator activity of the SUMO-deficient PARP1 mutant in genetically complemented K562 cells. Together with the fourth article (164), which describes PARP1 as co-activator of HIF-1α, these results provide evidence that PARP1 is a co-activator of hypoxia dependent gene expression and that posttranslational modifications of PARP1 regulate this activity.

In the second article we explored, based on the third article (see below), lysine residues as putative ADP-ribose acceptor amino acids. The four core histone tails were shown to be ADP-ribosylated by PARP1 *in vitro*. The site of modification was mapped by site directed mutagenesis to specific regions within the unstructured N-terminal tail of the histones. Mass spectrometry of histone H3 and H4 tail peptides revealed the PARP1-mediated ADP-ribosylation of H3K27, H3K37 and H4K16. An acetylated histone H4 peptide at K16 was a poor substrate for ADP-ribosylation. Furthermore, molecular dynamics of histone H3 and H4 tail, docked into the catalytic cleft of PARP1 revealed a stable interaction of H3K27 and H4K16 with the catalytically active glutamate of PARP1.

The third article established the molecular mechanism of poly(ADP-ribosylation) by PARP1 (165). The earlier reported glutamic acid residues in the auto-modification domain of PARP1 were dispensable for the auto-modification of PARP1. Instead, site directed mutagenesis at different lysine residues revealed that these lysines were ADP-ribose acceptor sites. Interestingly, the same lysines (K498, K521 and K524) were previously reported to be acetylated by p300 upon inflammatory stimuli.

The fifth article summarizes the current knowledge of ADP-ribosylation of histones in the form of a review. Beside PARP1, histones were the first proteins reported to be ADP-

ribosylated. The article reviews the existing literature on ADP-ribosylation of histones in light of our recent findings that lysines are the acceptor sites of ADP-ribose on histones.

4.2 Role of sumoylated PARP1 in the cell

In the presented work, it was found that SUMO-modification of PARP1 increased upon exposure of cells to hypoxia. Investigation of the functional role of SUMO-modified PARP1 focused on the transcriptional co-activator activity of PARP1 of hypoxia inducible transcription, since no effect of sumoylation was found for the DNA-binding ability of PARP1, Caspase 3 – mediated PARP1 cleavage or its poly(ADP-ribosyl)ation activity *in vitro*. Proteomic studies reported that PARP1 is poly-sumoylated after heat shock (78, 166). More specifically, Anne Dejean`s lab reported that PARP1 was poly-sumoylated at K203 and K486 after heat shock by the SUMO E3 ligase PIASy. Furthermore, SUMO-targeted ubiquitin ligase RNF4 recognized poly-sumoylated PARP1 and mediated heat-shock-inducible ubiquitination of PARP1, thus regulating the stability of PARP1 and the transcriptional function of PARP1 for heat-shock inducible genes in HeLa cells (167). In contrast to these reports, no poly-sumoylation of PARP1 after hypoxic induction was detected in our studies (163). Furthermore, we did not observe enhanced sumoylation of PARP1 upon overexpression of PIAS family members in HEK293T cells (data not shown). Instead, we reported only mono-sumoylation of PARP1, which was enhanced upon overexpression of HDAC4 in HEK293T cells (Fig. 4A), suggesting that HDAC4 acts as SUMO E3-ligase for mono-sumoylation of PARP1. Interestingly, the histone deacetylase activity of HDAC4 was dispensable for this effect, since enzymatically inactive, purified HDAC4 was still able to enhance sumoylation of PARP1 *in vitro* (Fig. 4B). The discrepancy between our results and the results from Anne Dejean`s group could be explained by the application of different stimuli (hypoxia vs. heat shock) and the usage of different cell lines (HEK293T vs. HeLa). For this work, only single genes were assayed using quantitative real-time PCR. While focusing on single genes allows fast and robust detection of mRNA levels, genomic approaches permit the observation of thousands of genes, thus enabling the researcher to analyze global gene expression and to find new target genes with the tested conditions. With the cell lines generated for this thesis, DNA-microarray studies could be carried out to investigate whether sumoylation deficient PARP1 mutant (K486R) only influences the expression of some specific hypoxia inducible genes or most of them. To investigate the contribution of PARP1`s SUMO-modification in a more physiological context, a knock-in mouse model would be of advantage. One could then compare wild-type mice with PARP1-

sumoylation deficient mice (K486R) in mouse models of cancer formation and -progression, inflammation or metabolic disorders.

Sumoylation and poly(ADP-ribosyl)ation of PARP1 can co-exist on the same PARP1 molecule. It is therefore tempting to speculate that classical PARP1 activation by genotoxic stress might at the same time involve sumoylation. During initiation of base excision repair (BER), poly(ADP-ribosyl)ated PARP1 was described to recruit the scaffolding protein XRCC1 to the site of DNA-damage, thus facilitating DNA-repair signalling *in vivo* (105). XRCC1 possesses a SUMO-interacting motif, and is itself SUMO-modified (personal communication D. Schürmann and (71)). Thus, sumoylation of PARP1 could influence duration and strength of the PARP1 interaction with XRCC1. Additionally, upon gamma-irradiation poly(ADP-ribosyl)ated PARP1 was shown to recruit PIASy, IKKγ (NEMO) and ATM (168). Activated PARP1 and PIASy mediated IKKγ sumoylation, which in turn permitted IKK and NF-κB activation. Since signalosome formation depended on poly(ADP-ribosyl)ated PARP1, one could speculate that the signalosome formation would be facilitated, if PARP1 is additionally SUMO-modified and able to recruit PIASy by its SUMO-interacting motif (169)

Posttranslational modifications of other PARP-family members, such as PARP2 are less well characterized. However, a proteomic screen suggested that PARP2 is poly-sumoylated after heat shock (78). Indeed, PARP2 was sumoylated by SUMO3 *in vitro* also in our hands (Fig. 9). The SUMO-modification of PARP2 was completely abolished in the absence of the WGR domain of PARP2, harbouring only one putative SUMO-consensus site at lysine 143. One could confirm by site directed mutagenesis, whether this lysine is a real acceptor site for SUMO-modification *in vitro* and *in vivo* and investigate the functional role of sumoylation of PARP2 (e.g. after heat shock).

4.3 Crosstalk of acetylation and ADP-ribosylation

The acetylation of PARP1 by p300/CPB at different lysine residues (K498, K505, K508, K521, K524) was reported to be necessary for the binding of PARP1 to p50 NF-κB subunit and for synergistic co-activation of NF-κB dependent gene expression (137). We observed that the same lysines (K498, K521, K524) are ADP-ribosylated, implicating an interesting crosstalk between ADP-ribosylation and acetylation. Remarkably, the enzymatic activity of PARP1 was not required for transcriptional co-activation of NF-κB (170). Thus acetylated PARP1 could potentially be less active under conditions when the ADP-ribose acceptor sites are already occupied by an acetyl-group. Thus, it might be that deacetylating enzymes, such as sirtuins, regulate the activity of PARP1 through the removal of acetyl groups. A

cross-talk between SIRT1 and PARP1 was already reported (171). SIRT1$^{-/-}$ cells were reported to have higher PARP1 activity than wild type cells, providing a functional link between two NAD^+-dependent pathways. Along this line, it was shown that PARP1 acetylation was increased in SIRT1$^{-/-}$ cells and that acetylated recombinant PARP1 had higher enzymatic activity than unmodified PARP1 (172). Deacetylation of PARP1 by SIRT1 blocked the enzymatic activity and protected against PARP1-mediated cell death in cardiomyocytes. In the report of Rajamohan and co-workers most of the analysis was performed with the histone acetyltransferase PCAF, rather than p300. Thus, it could be that other lysine residues than K498, K505, K508, K521 and K524 were acetylated under the tested conditions, still enabling acetylated PARP1 to auto-ADP-ribosylate K498, K521 and K524. We observed acetylation of K105 and K108 within the first zinc finger domain of PARP1 by PCAF *in vitro* (Fig. 7 and 8). Thus, one could speculate that the acetylation of the first zinc finger stimulates the enzymatic activity of PARP1, which would explain the synergy between acetylation and PARP1 activity (172).

Interestingly, sirtuins were able to deacetylate PARP1 in a NAD^+ dependent manner *in vitro* (Fig. 6). Western blot was performed with a monoclonal anti-acetyl PARP1 antibody (anti-E4), which recognizes acetylated K498, K505 and K508. SIRT1, SIRT2 and SIRT6, but not SIRT7 were able to reverse p300-mediated PARP1 acetylation of the above mentioned lysines. We can currently not exclude that SIRTs also mediate deacetylation of other acetylated lysines of PARP1 (e.g. in the DNA-binding domain).

It was previously reported that HDAC1, 2 and HDAC3 mediate PARP1 deacetylation (137). In this work we tested, whether HDAC4 could deacetylate PARP1. Deacetylation of PARP1 was observed only after immunoprecipitation of HDAC4 (Fig. 5B), but not by incubation of acetylated PARP1 with recombinant HDAC4 (Fig. 5A). Therefore, it is possible that HDAC4 is in complex with other histone deacetylases *in vivo*, which are able to deacetylate PARP1. In line with our own results, the enzymatic activity of HDAC4 was shown to depend on the association with the HDAC3/SMRT/N-CoR complex, thus suggesting that HDAC4 is not an PARP1 deacetylase, but operates by recruiting preexisting enzymatically active HDAC3 protein complexes (90). Therefore, it is possible that acetyl-groups of PARP1 are removed by the HDAC3/HDAC4/SMRT/N-CoR complex, which abrogate the transcriptional co-activator functions of PARP1. After deacetylation of PARP1, HDAC4 might facilitate sumoylation of PARP1, which leads to dissociation of PARP1 from the promoter and "tags" the protein as an already used co-activator. During this time, PARP1 is ready to auto-ADP-ribosylate itself again, since sumoylated PARP1 inhibits p300-mediated acetylation of K498, K505 and K508 (163), which are targets for auto-ADP-ribosylation (165). Moreover, SUMO proteases SENP1 or SENP3 might cleave off the SUMO moiety from PARP1 and render PARP1 ready for another cycle of transcriptional co-activation.

Finally, poly(ADP-ribosyl)ated PARP1 might affect the acetylation status of other proteins also by an indirect manner. Class III histone deacetylases (sirtuins) share NAD^+ as substrate with PARP1. This provides a link between ADP-ribosylation and deacetylation, as proposed by Zhang (173). Since deacetylation of histones is thought to correlate with attenuation of gene expression, competition between sirtuins and activated PARP1 for the cellular NAD^+ pool could lead to inhibition of deacetylation and thus activation of gene expression.

4.4 Identification of ADP-ribosylated residues by mass spectrometry

Novel mass spectrometric methods enabled the discoverage of lysine ADP-ribosylation (see 2nd manuscript). Application of the electron transfer dissociation (ETD) fragmentation technique, which leaves unstable posttranslational modifications intact, allowed us to assign intact ADP-ribose to specific amino acids. Positively charged peptides are fragmented much better by ETD than by conventional collision-induced-decay (CID) technique. ETD thus facilitated the fragmentation of highly positively charged histone peptides with their corresponding modification. However, for the fragmentation of less charged peptides, this preference might be a drawback. Therefore, a combination of ETD and CID fragmentation techniques would be of advantage. The invention of "de-novo sequencing" of proteins from complex samples by the usage of the protease Lys-N, which cleaves the peptide backbone N-terminally of the basic residue lysine, together with the ETD fragmentation technology might boost our knowledge of so far unrecognized posttranslational modifications (174). Together with novel enrichment methods of ADP-ribosylated proteins, ETD might allow to study protein ADP-ribosylation in a systems-biological approach.

4.5 A new letter for the histone code

In the 1980s ADP-ribose acceptor sites were detected by biochemical approaches in histones (175, 176). Ogata and colleagues identified glutamic acid residues in histone H1 and histone H2B to be modified when they incubated chromatin from rat liver with radioactive NAD^+. The enzyme responsible for the modification was, however, never identified. Thus it is possible that PARP1 or other PARP-family members or even unrelated ADP-ribosyl-transferases are responsible for the modification of the identified glutamates. Moreover, the confirmation of glutamic acid residues as ADP-ribose acceptor sites by site directed mutagenesis or mass spectrometry is still missing.

In contrast, we observed that poly-L-lysine and poly-L-arginine peptides were ADP-ribosylated by PARP1 (2nd manuscript). This led to the investigation of histones as ADP-ribose acceptor proteins. We found that core histone tails H2A, H2B, H3 and H4 are ADP-ribosylated. Of note, the histone tails of H2A, H3 and H4 do not contain a single glutamic acid residue, but are rich in the positively charged amino acids lysine and arginine. Importantly, we observed no reduction in the modification of the H2B tail by recombinant purified PARP1 when we mutated the single glutamate at position 2 of H2B to an alanine. We mapped subsequently the domain important for interaction and ADP-ribosylation for each tail and mutated different residues (see Dissertation Matthias Altmeyer). In summary, the most important residues for ADP-ribosylation of the H2A-tail were K13 and K15 and for the H2B-tail several lysine and/or arginine residues between K27 and K34. For the H3-tail, K23/27 and K36/37, as well as K16 and K20 for the H4-tail were identified to be important for ADP-ribosylation. Mass spectrometric analysis confirmed ADP-ribosylation of H3K27 and H3K37 and H4K16. Whether ADP-ribosylation co-exists with other histone modifications on nucleosomes remains to be determined. The particular combinations of posttranslational modifications have been suggested to constitute a histone code that influences chromatin function by creating or removing binding sites for chromatin-associated proteins. Therefore, the newly described lysine ADP-ribosylation could thus represent a novel letter in the complex language of histone modifications and extent the number of different PTM combinations.

4.6 Histone ADP-ribosylation as a stable mark?

In general, poly(ADP-ribosyl)ation is a transient and reversible process. Poly(ADP-ribose) is degraded by the nuclear PARG enzyme, as well as by the cytoplasmic ARH family of ADP-ribose protein hydrolases (112, 114, 177). From the nature of the chemical linkage, on would have to assume that lysine-(ADP-ribosyl)ation is not removed by the known ADP-ribose hydrolyzing enzymes. Therefore it will be important to analyse PAR degrading enzymes in regard to their ability to degrade poly(ADP-ribosyl)ated histone tails. It will be important to distinguish, whether ADP-ribosylation of lysines is fully reversible, or remains as stable mark. A stable histone tail mark, which could only be removed by replacing the affected histone with a non-marked histone, could be part of a chromatin memory and may function as epigenetic mechanism.

4.7 Readers of poly(ADP-ribose)

Posttranslational histone modifications are often recognized by histone-binding modules (effectors). For example, chromodomain, tudor-domain, plant homeodomain (PHD), malignant brain tumor motif (MBT) as well as ankyrin repeats recognize methylated lysine and arginine residues (16). Acetylation marks, in contrast, are read by bromodomains, and phosphorylation is recognized by a domain within 14-3-3 proteins (15). PARP1-generated poly(ADP-ribose) was already described to be recognized by at least three different protein motifs, basic PAR binding motif (143), a PAR binding zinc fingers (178), and by macrodomains (179). Interestingly, macroH2A was shown to be recruited to sites of DNA-damage, where it interacted with poly(ADP-ribose) (147). The chromatin remodeler Alc1 contains a macro-domain that bound to poly(ADP-ribosyl)ated PARP1 upon DNA-damage (180, 181). Interaction of Alc1 with poly(ADP-ribosyl)ated PARP1 activated the ATPase activity of Alc1 and promoted chromatin remodeling. The activity of Alc1 is dependent on the basic H4K16-K20 batch (180). Thus one can speculate that the ATPase activity of Alc1 could be additionally regulated by the presence of ADP-ribosylated H4K16. However, whether Alc1 or other proteins, which harbour these PAR sensing domains would be recruited *in vivo* to poly(ADP-ribosyl)ated histones, was not yet tested.

4.8 PARP1 and Histone H4

Histone H4 is heavily posttranslationally modified by acetylation and methylation (15). For example, H4K16 is acetylated by Tip60 and by ScSAS2 (SpMST2) (15). Deacetylation of H4K16 is regulated by SIRT2 (182). Whether ADP-ribosylation of histones is co-inciding or mutually exclusive with other histone modifications is yet not clear. Some reports found that poly(ADP-ribosylation) of histone H4 co-incided with acetylated H4 (183, 184). However, we observed that acetylation of H4K16 inhibited ADP-ribosylation by PARP1 *in vitro*. Therefore, direct competition of ADP-ribosylation with acetylation for the same lysine residue might be also relevant *in vivo*. To further investigate this possibility, the generation of antibodies raised against ADP-ribosylated histones will certainly allow the analysis of histone ADP-ribosylation and the detection of putative crosstalks with other histone modifications *in vivo*.

One could imagine that ADP-ribosylation of H4K16 repels histone acetyl-transferase complexes from H4K16 possibly by steric hindrance or by the highly negative charge of the ADP-ribose moiety.

The acetylation of H4K16 prevents the formation of the 30nm chromatin fiber and the generation of higher order chromatin structures by inhibiting internucleosomal contacts of the basic H4K16-K20 batch with the two acidic patches on the carboxy-terminal α-helices of histone H2A (185). Moreover, the generation of higher order chromatin structures could be affected by ADP-ribosylated H4K16. Acetylation of H4K16 is often described as switch, altering chromatin topology but also repelling various non-histone modulators. For example, the ISWI-containing ATP-dependent chromatin remodeller ACF contributes to high-order chromatin folding by regularly spacing nucleosomes. But solely histones in the absence of histone H4K16 acetylation are remodelled by ACF (186). Therefore, it could be expected that ADP-ribosylation of H4K16 inhibits binding of ACF and impedes chromatin remodeling.

In regard of charge and structure of poly(ADP-ribose) one would expect that poly(ADP-ribosyl)ation of histone tails leads to decompaction of heterochromatin and/or prevent free histones from being integrated into nucleosomes. Both processes may be especially important during replication, where heterochromatin needs to be decondensed and new nucleosomes have to be incorporated into the chromatin.

In summary, ADP-ribosylation of histones by PARP1 occurs as posttranslational modification at distinct lysine residues. The implications for processes such as nucleosome formation, regulation of higher order chromatin structure, histone degradation, histone shuttling and epigenetics remain to be determined.

5. References

1. Lodish, H., Berk, A., Kaiser, C. A., Krieger, M., Scott, M. P., Bretscher, A., Ploegh, H., and Matsudaira, P. (1999) *Molecular Cell Biology*, W.H. Freeman & Co, New York
2. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**, 251-260
3. Wolffe, A. P. (1997) Histone H1. *Int J Biochem Cell Biol* **29**, 1463-1466
4. Polo, S. E., and Almouzni, G. (2006) Chromatin assembly: a basic recipe with various flavours. *Curr Opin Genet Dev* **16**, 104-111
5. Felsenfeld, G., and Groudine, M. (2003) Controlling the double helix. *Nature* **421**, 448-453
6. Saha, A., Wittmeyer, J., and Cairns, B. R. (2006) Chromatin remodelling: the industrial revolution of DNA around histones. *Nat Rev Mol Cell Biol* **7**, 437-447
7. Kimura, H., and Cook, P. R. (2001) Kinetics of core histones in living human cells: little exchange of H3 and H4 and some rapid exchange of H2B. *J Cell Biol* **153**, 1341-1353
8. Adams, C. C., and Workman, J. L. (1995) Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative. *Mol Cell Biol* **15**, 1405-1421
9. Phelan, M. L., Schnitzler, G. R., and Kingston, R. E. (2000) Octamer transfer and creation of stably remodeled nucleosomes by human SWI-SNF and its isolated ATPases. *Mol Cell Biol* **20**, 6380-6389
10. Kireeva, M. L., Walter, W., Tchernajenko, V., Bondarenko, V., Kashlev, M., and Studitsky, V. M. (2002) Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription. *Mol Cell* **9**, 541-552
11. Chen, H., Li, B., and Workman, J. L. (1994) A histone-binding protein, nucleoplasmin, stimulates transcription factor binding to nucleosomes and factor-induced nucleosome disassembly. *EMBO J* **13**, 380-390
12. Lorch, Y., Maier-Davis, B., and Kornberg, R. D. (2006) Chromatin remodeling by nucleosome disassembly in vitro. *Proc Natl Acad Sci U S A* **103**, 3090-3093
13. Swaminathan, V., Kishore, A. H., Febitha, K. K., and Kundu, T. K. (2005) Human histone chaperone nucleophosmin enhances acetylation-dependent chromatin transcription. *Mol Cell Biol* **25**, 7534-7545
14. Walter, P. P., Owen-Hughes, T. A., Cote, J., and Workman, J. L. (1995) Stimulation of transcription factor binding and histone displacement by nucleosome assembly protein 1 and nucleoplasmin requires disruption of the histone octamer. *Mol Cell Biol* **15**, 6178-6187
15. Kouzarides, T. (2007) Chromatin modifications and their function. *Cell* **128**, 693-705
16. Campos, E. I., and Reinberg, D. (2009) Histones: annotating chromatin. *Annu Rev Genet* **43**, 559-599
17. Strahl, B. D., and Allis, C. D. (2000) The language of covalent histone modifications. *Nature* **403**, 41-45
18. Spotswood, H. T., and Turner, B. M. (2002) An increasingly complex code. *J Clin Invest* **110**, 577-582
19. de la Cruz, X., Lois, S., Sanchez-Molina, S., and Martinez-Balbas, M. A. (2005) Do protein motifs read the histone code? *Bioessays* **27**, 164-175
20. Sarma, K., and Reinberg, D. (2005) Histone variants meet their match. *Nat Rev Mol Cell Biol* **6**, 139-149
21. McKittrick, E., Gafken, P. R., Ahmad, K., and Henikoff, S. (2004) Histone H3.3 is enriched in covalent modifications associated with active chromatin. *Proc Natl Acad Sci U S A* **101**, 1525-1530
22. Bassing, C. H., Suh, H., Ferguson, D. O., Chua, K. F., Manis, J., Eckersdorff, M., Gleason, M., Bronson, R., Lee, C., and Alt, F. W. (2003) Histone H2AX: a dosage-dependent suppressor of oncogenic translocations and tumors. *Cell* **114**, 359-370
23. Celeste, A., Difilippantonio, S., Difilippantonio, M. J., Fernandez-Capetillo, O., Pilch, D. R., Sedelnikova, O. A., Eckhaus, M., Ried, T., Bonner, W. M., and Nussenzweig, A. (2003) H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. *Cell* **114**, 371-383
24. Stucki, M., and Jackson, S. P. (2006) gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst)* **5**, 534-543

25. Doyen, C. M., An, W., Angelov, D., Bondarenko, V., Mietton, F., Studitsky, V. M., Hamiche, A., Roeder, R. G., Bouvet, P., and Dimitrov, S. (2006) Mechanism of polymerase II transcription repression by the histone variant macroH2A. *Mol Cell Biol* **26**, 1156-1164
26. Guillemette, B., Bataille, A. R., Gevry, N., Adam, M., Blanchette, M., Robert, F., and Gaudreau, L. (2005) Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning. *PLoS Biol* **3**, e384
27. Li, B., Pattenden, S. G., Lee, D., Gutierrez, J., Chen, J., Seidel, C., Gerton, J., and Workman, J. L. (2005) Preferential occupancy of histone variant H2AZ at inactive promoters influences local histone modifications and chromatin remodeling. *Proc Natl Acad Sci U S A* **102**, 18385-18390
28. Zhang, H., Roberts, D. N., and Cairns, B. R. (2005) Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. *Cell* **123**, 219-231
29. Schwartz, B. E., and Ahmad, K. (2005) Transcriptional activation triggers deposition and removal of the histone variant H3.3. *Genes Dev* **19**, 804-814
30. Henikoff, S., and Ahmad, K. (2005) Assembly of variant histones into chromatin. *Annu Rev Cell Dev Biol* **21**, 133-153
31. Orphanides, G., and Reinberg, D. (2002) A unified theory of gene expression. *Cell* **108**, 439-451
32. Perkins, N. D. (2006) Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene* **25**, 6717-6730
33. Kaluz, S., Kaluzova, M., and Stanbridge, E. J. (2008) Regulation of gene expression by hypoxia: integration of the HIF-transduced hypoxic signal at the hypoxia-responsive element. *Clin Chim Acta* **395**, 6-13
34. Remenyi, A., Scholer, H. R., and Wilmanns, M. (2004) Combinatorial control of gene expression. *Nat Struct Mol Biol* **11**, 812-815
35. Li, B., Carey, M., and Workman, J. L. (2007) The role of chromatin during transcription. *Cell* **128**, 707-719
36. Goodman, R. H., and Smolik, S. (2000) CBP/p300 in cell growth, transformation, and development. *Genes Dev* **14**, 1553-1577
37. Chan, H. M., and La Thangue, N. B. (2001) p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci* **114**, 2363-2373
38. Ouyang, J., Shi, Y., Valin, A., Xuan, Y., and Gill, G. (2009) Direct binding of CoREST1 to SUMO-2/3 contributes to gene-specific repression by the LSD1/CoREST1/HDAC complex. *Mol Cell* **34**, 145-154
39. Lopez-Barneo, J., Pardal, R., and Ortega-Saenz, P. (2001) Cellular mechanism of oxygen sensing. *Annu Rev Physiol* **63**, 259-287
40. Brown, J. M. (2000) Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. *Mol Med Today* **6**, 157-162
41. Nizet, V., and Johnson, R. S. (2009) Interdependence of hypoxic and innate immune responses. *Nat Rev Immunol* **9**, 609-617
42. Semenza, G. L. (2009) Regulation of oxygen homeostasis by hypoxia-inducible factor 1. *Physiology (Bethesda)* **24**, 97-106
43. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A* **92**, 5510-5514
44. Bruick, R. K., and McKnight, S. L. (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* **294**, 1337-1340
45. Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54
46. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. (2002) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* **295**, 858-861
47. Freedman, S. J., Sun, Z. Y., Poy, F., Kung, A. L., Livingston, D. M., Wagner, G., and Eck, M. J. (2002) Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha. *Proc Natl Acad Sci U S A* **99**, 5367-5372

48. Blagosklonny, M. V., An, W. G., Romanova, L. Y., Trepel, J., Fojo, T., and Neckers, L. (1998) p53 inhibits hypoxia-inducible factor-stimulated transcription. *J Biol Chem* **273**, 11995-11998
49. Xenaki, G., Ontikatze, T., Rajendran, R., Stratford, I. J., Dive, C., Krstic-Demonacos, M., and Demonacos, C. (2008) PCAF is an HIF-1alpha cofactor that regulates p53 transcriptional activity in hypoxia. *Oncogene* **27**, 5785-5796
50. Ulrich, H. D. (2007) SUMO teams up with ubiquitin to manage hypoxia. *Cell* **131**, 446-447
51. Papandreou, I., Cairns, R. A., Fontana, L., Lim, A. L., and Denko, N. C. (2006) HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab* **3**, 187-197
52. Chandel, N. S., and Budinger, G. R. (2007) The cellular basis for diverse responses to oxygen. *Free Radic Biol Med* **42**, 165-174
53. Supuran, C. T. (2008) Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov* **7**, 168-181
54. Kaluz, S., Kaluzova, M., Liao, S. Y., Lerman, M., and Stanbridge, E. J. (2009) Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: A one transcription factor (HIF-1) show? *Biochim Biophys Acta* **1795**, 162-172
55. Erler, J. T., Bennewith, K. L., Nicolau, M., Dornhofer, N., Kong, C., Le, Q. T., Chi, J. T., Jeffrey, S. S., and Giaccia, A. J. (2006) Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* **440**, 1222-1226
56. Lucero, H. A., and Kagan, H. M. (2006) Lysyl oxidase: an oxidative enzyme and effector of cell function. *Cell Mol Life Sci* **63**, 2304-2316
57. Akiri, G., Sabo, E., Dafni, H., Vadasz, Z., Kartvelishvily, Y., Gan, N., Kessler, O., Cohen, T., Resnick, M., Neeman, M., and Neufeld, G. (2003) Lysyl oxidase-related protein-1 promotes tumor fibrosis and tumor progression in vivo. *Cancer Res* **63**, 1657-1666
58. Payne, S. L., Hendrix, M. J., and Kirschmann, D. A. (2007) Paradoxical roles for lysyl oxidases in cancer--a prospect. *J Cell Biochem* **101**, 1338-1354
59. Peinado, H., Moreno-Bueno, G., Hardisson, D., Perez-Gomez, E., Santos, V., Mendiola, M., de Diego, J. I., Nistal, M., Quintanilla, M., Portillo, F., and Cano, A. (2008) Lysyl oxidase-like 2 as a new poor prognosis marker of squamous cell carcinomas. *Cancer Res* **68**, 4541-4550
60. Seet, B. T., Dikic, I., Zhou, M. M., and Pawson, T. (2006) Reading protein modifications with interaction domains. *Nat Rev Mol Cell Biol* **7**, 473-483
61. Meinnel, T., and Giglione, C. (2008) Protein lipidation meets proteomics. *Front Biosci* **13**, 6326-6340
62. Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008) Glycosyltransferases: structures, functions, and mechanisms. *Annu Rev Biochem* **77**, 521-555
63. Staub, O. (2004) Ubiquitylation and isgylation: overlapping enzymatic cascades do the job. *Sci STKE* **2004**, pe43
64. Geiss-Friedlander, R., and Melchior, F. (2007) Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* **8**, 947-956
65. Rabut, G., and Peter, M. (2008) Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series. *EMBO Rep* **9**, 969-976
66. Gyorgy, B., Toth, E., Tarcsa, E., Falus, A., and Buzas, E. I. (2006) Citrullination: a posttranslational modification in health and disease. *Int J Biochem Cell Biol* **38**, 1662-1677
67. Davidson, H. W., Rhodes, C. J., and Hutton, J. C. (1988) Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases. *Nature* **333**, 93-96
68. Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., and Becker, J. (1998) Structure determination of the small ubiquitin-related modifier SUMO-1. *J Mol Biol* **280**, 275-286
69. Guo, D., Li, M., Zhang, Y., Yang, P., Eckenrode, S., Hopkins, D., Zheng, W., Purohit, S., Podolsky, R. H., Muir, A., Wang, J., Dong, Z., Brusko, T., Atkinson, M., Pozzilli, P., Zeidler, A., Raffel, L. J., Jacob, C. O., Park, Y., Serrano-Rios, M., Larrad, M. T., Zhang, Z., Garchon, H. J., Bach, J. F., Rotter, J. I., She, J. X., and Wang, C. Y. (2004) A functional variant of SUMO4, a new I kappa B alpha modifier, is associated with type 1 diabetes. *Nat Genet* **36**, 837-841
70. Saitoh, H., and Hinchey, J. (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem* **275**, 6252-6258

71. Rosas-Acosta, G., Russell, W. K., Deyrieux, A., Russell, D. H., and Wilson, V. G. (2005) A universal strategy for proteomic studies of SUMO and other ubiquitin-like modifiers. *Mol Cell Proteomics* **4**, 56-72
72. Vertegaal, A. C., Andersen, J. S., Ogg, S. C., Hay, R. T., Mann, M., and Lamond, A. I. (2006) Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics. *Mol Cell Proteomics* **5**, 2298-2310
73. Dasso, M. (2008) Emerging roles of the SUMO pathway in mitosis. *Cell Div* **3**, 5
74. Johnson, E. S. (2004) Protein modification by SUMO. *Annu Rev Biochem* **73**, 355-382
75. Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A., and Sistonen, L. (2006) PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A* **103**, 45-50
76. Pichler, A., Knipscheer, P., Oberhofer, E., van Dijk, W. J., Korner, R., Olsen, J. V., Jentsch, S., Melchior, F., and Sixma, T. K. (2005) SUMO modification of the ubiquitin-conjugating enzyme E2-25K. *Nat Struct Mol Biol* **12**, 264-269
77. Hecker, C. M., Rabiller, M., Haglund, K., Bayer, P., and Dikic, I. (2006) Specification of SUMO1- and SUMO2-interacting motifs. *J Biol Chem* **281**, 16117-16127
78. Golebiowski, F., Matic, I., Tatham, M. H., Cole, C., Yin, Y., Nakamura, A., Cox, J., Barton, G. J., Mann, M., and Hay, R. T. (2009) System-wide changes to SUMO modifications in response to heat shock. *Sci Signal* **2**, ra24
79. Comerford, K. M., Leonard, M. O., Karhausen, J., Carey, R., Colgan, S. P., and Taylor, C. T. (2003) Small ubiquitin-related modifier-1 modification mediates resolution of CREB-dependent responses to hypoxia. *Proc Natl Acad Sci U S A* **100**, 986-991
80. Shao, R., Zhang, F. P., Tian, F., Anders Friberg, P., Wang, X., Sjoland, H., and Billig, H. (2004) Increase of SUMO-1 expression in response to hypoxia: direct interaction with HIF-1alpha in adult mouse brain and heart in vivo. *FEBS Lett* **569**, 293-300
81. Carbia-Nagashima, A., Gerez, J., Perez-Castro, C., Paez-Pereda, M., Silberstein, S., Stalla, G. K., Holsboer, F., and Arzt, E. (2007) RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1alpha during hypoxia. *Cell* **131**, 309-323
82. Bae, S. H., Jeong, J. W., Park, J. A., Kim, S. H., Bae, M. K., Choi, S. J., and Kim, K. W. (2004) Sumoylation increases HIF-1alpha stability and its transcriptional activity. *Biochem Biophys Res Commun* **324**, 394-400
83. Cheng, J., Kang, X., Zhang, S., and Yeh, E. T. (2007) SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell* **131**, 584-595
84. Huang, C., Han, Y., Wang, Y., Sun, X., Yan, S., Yeh, E. T., Chen, Y., Cang, H., Li, H., Shi, G., Cheng, J., Tang, X., and Yi, J. (2009) SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation. *EMBO J* **28**, 2748-2762
85. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* **325**, 834-840
86. Yang, X. J. (2004) Lysine acetylation and the bromodomain: a new partnership for signaling. *Bioessays* **26**, 1076-1087
87. Yang, X. J. (2004) The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. *Nucleic Acids Res* **32**, 959-976
88. Sun, Y., Jiang, X., Xu, Y., Ayrapetov, M. K., Moreau, L. A., Whetstine, J. R., and Price, B. D. (2009) Histone H3 methylation links DNA damage detection to activation of the tumour suppressor Tip60. *Nat Cell Biol* **11**, 1376-1382
89. Sapountzi, V., Logan, I. R., and Robson, C. N. (2006) Cellular functions of TIP60. *Int J Biochem Cell Biol* **38**, 1496-1509
90. Gallinari, P., Di Marco, S., Jones, P., Pallaoro, M., and Steinkuhler, C. (2007) HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics. *Cell Res* **17**, 195-211
91. Yang, X. J., and Seto, E. (2003) Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. *Curr Opin Genet Dev* **13**, 143-153
92. Blander, G., and Guarente, L. (2004) The Sir2 family of protein deacetylases. *Annu Rev Biochem* **73**, 417-435
93. Finkel, T., Deng, C. X., and Mostoslavsky, R. (2009) Recent progress in the biology and physiology of sirtuins. *Nature* **460**, 587-591
94. Kawahara, T. L., Michishita, E., Adler, A. S., Damian, M., Berber, E., Lin, M., McCord, R. A., Ongaigui, K. C., Boxer, L. D., Chang, H. Y., and Chua, K. F. (2009) SIRT6 links histone H3

lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* **136**, 62-74
95. Liszt, G., Ford, E., Kurtev, M., and Guarente, L. (2005) Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase. *J Biol Chem* **280**, 21313-21320
96. Sapetschnig, A., Rischitor, G., Braun, H., Doll, A., Schergaut, M., Melchior, F., and Suske, G. (2002) Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *EMBO J* **21**, 5206-5215
97. Bouras, T., Fu, M., Sauve, A. A., Wang, F., Quong, A. A., Perkins, N. D., Hay, R. T., Gu, W., and Pestell, R. G. (2005) SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1. *J Biol Chem* **280**, 10264-10276
98. Bossis, G., and Melchior, F. (2006) SUMO: regulating the regulator. *Cell Div* **1**, 13
99. Shalizi, A., Gaudilliere, B., Yuan, Z., Stegmuller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J. W., and Bonni, A. (2006) A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. *Science* **311**, 1012-1017
100. Ghisletti, S., Huang, W., Ogawa, S., Pascual, G., Lin, M. E., Willson, T. M., Rosenfeld, M. G., and Glass, C. K. (2007) Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma. *Mol Cell* **25**, 57-70
101. Gregoire, S., and Yang, X. J. (2005) Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. *Mol Cell Biol* **25**, 2273-2287
102. Stankovic-Valentin, N., Deltour, S., Seeler, J., Pinte, S., Vergoten, G., Guerardel, C., Dejean, A., and Leprince, D. (2007) An acetylation/deacetylation-SUMOylation switch through a phylogenetically conserved psiKXEP motif in the tumor suppressor HIC1 regulates transcriptional repression activity. *Mol Cell Biol* **27**, 2661-2675
103. Zhao, X., Sternsdorf, T., Bolger, T. A., Evans, R. M., and Yao, T. P. (2005) Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. *Mol Cell Biol* **25**, 8456-8464
104. Hassa, P. O., Haenni, S. S., Elser, M., and Hottiger, M. O. (2006) Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol Mol Biol Rev* **70**, 789-829
105. D'Amours, D., Desnoyers, S., D'Silva, I., and Poirier, G. G. (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J* **342 (Pt 2)**, 249-268
106. Langelier, M. F., Servent, K. M., Rogers, E. E., and Pascal, J. M. (2008) A third zinc-binding domain of human poly(ADP-ribose) polymerase-1 coordinates DNA-dependent enzyme activation. *J Biol Chem* **283**, 4105-4114
107. Rodriguez, M. C., and Songyang, Z. (2008) BRCT domains: phosphopeptide binding and signaling modules. *Front Biosci* **13**, 5905-5915
108. Hassa, P. O., and Hottiger, M. O. (2008) The diverse biological roles of mammalian PARPS, a small but powerful family of poly-ADP-ribose polymerases. *Front Biosci* **13**, 3046-3082
109. Ruf, A., Mennissier de Murcia, J., de Murcia, G., and Schulz, G. E. (1996) Structure of the catalytic fragment of poly(AD-ribose) polymerase from chicken. *Proc Natl Acad Sci U S A* **93**, 7481-7485
110. Schreiber, V., Dantzer, F., Ame, J. C., and de Murcia, G. (2006) Poly(ADP-ribose): novel functions for an old molecule. *Nat Rev Mol Cell Biol* **7**, 517-528
111. Cohen-Armon, M., Visochek, L., Rozensal, D., Kalal, A., Geistrikh, I., Klein, R., Bendetz-Nezer, S., Yao, Z., and Seger, R. (2007) DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation. *Mol Cell* **25**, 297-308
112. Takada, T., Iida, K., and Moss, J. (1993) Cloning and site-directed mutagenesis of human ADP-ribosylarginine hydrolase. *J Biol Chem* **268**, 17837-17843
113. Ono, T., Kasamatsu, A., Oka, S., and Moss, J. (2006) The 39-kDa poly(ADP-ribose) glycohydrolase ARH3 hydrolyzes O-acetyl-ADP-ribose, a product of the Sir2 family of acetyl-histone deacetylases. *Proc Natl Acad Sci U S A* **103**, 16687-16691
114. Oka, S., Kato, J., and Moss, J. (2006) Identification and characterization of a mammalian 39-kDa poly(ADP-ribose) glycohydrolase. *J Biol Chem* **281**, 705-713
115. Masutani, M., Nozaki, T., Nishiyama, E., Shimokawa, T., Tachi, Y., Suzuki, H., Nakagama, H., Wakabayashi, K., and Sugimura, T. (1999) Function of poly(ADP-ribose) polymerase in response to DNA damage: gene-disruption study in mice. *Mol Cell Biochem* **193**, 149-152

116. Wang, Z. Q., Auer, B., Stingl, L., Berghammer, H., Haidacher, D., Schweiger, M., and Wagner, E. F. (1995) Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. *Genes Dev* **9**, 509-520
117. Shall, S., and de Murcia, G. (2000) Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? *Mutat Res* **460**, 1-15
118. Eliasson, M. J., Sampei, K., Mandir, A. S., Hurn, P. D., Traystman, R. J., Bao, J., Pieper, A., Wang, Z. Q., Dawson, T. M., Snyder, S. H., and Dawson, V. L. (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat Med* **3**, 1089-1095
119. de Groot, H., and Rauen, U. (2007) Ischemia-reperfusion injury: processes in pathogenetic networks: a review. *Transplant Proc* **39**, 481-484
120. Szabo, G., Liaudet, L., Hagl, S., and Szabo, C. (2004) Poly(ADP-ribose) polymerase activation in the reperfused myocardium. *Cardiovasc Res* **61**, 471-480
121. Burkart, V., Wang, Z. Q., Radons, J., Heller, B., Herceg, Z., Stingl, L., Wagner, E. F., and Kolb, H. (1999) Mice lacking the poly(ADP-ribose) polymerase gene are resistant to pancreatic beta-cell destruction and diabetes development induced by streptozocin. *Nat Med* **5**, 314-319
122. Pieper, A. A., Brat, D. J., Krug, D. K., Watkins, C. C., Gupta, A., Blackshaw, S., Verma, A., Wang, Z. Q., and Snyder, S. H. (1999) Poly(ADP-ribose) polymerase-deficient mice are protected from streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* **96**, 3059-3064
123. Junod, A., Lambert, A. E., Orci, L., Pictet, R., Gonet, A. E., and Renold, A. E. (1967) Studies of the diabetogenic action of streptozotocin. *Proc Soc Exp Biol Med* **126**, 201-205
124. Oliver, F. J., Menissier-de Murcia, J., Nacci, C., Decker, P., Andriantsitohaina, R., Muller, S., de la Rubia, G., Stoclet, J. C., and de Murcia, G. (1999) Resistance to endotoxic shock as a consequence of defective NF-kappaB activation in poly (ADP-ribose) polymerase-1 deficient mice. *EMBO J* **18**, 4446-4454
125. Kuhnle, S., Nicotera, P., Wendel, A., and Leist, M. (1999) Prevention of endotoxin-induced lethality, but not of liver apoptosis in poly(ADP-ribose) polymerase-deficient mice. *Biochem Biophys Res Commun* **263**, 433-438
126. Krishnakumar, R., Gamble, M. J., Frizzell, K. M., Berrocal, J. G., Kininis, M., and Kraus, W. L. (2008) Reciprocal binding of PARP-1 and histone H1 at promoters specifies transcriptional outcomes. *Science* **319**, 819-821
127. Ju, B. G., Lunyak, V. V., Perissi, V., Garcia-Bassets, I., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (2006) A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription. *Science* **312**, 1798-1802
128. Perillo, B., Ombra, M. N., Bertoni, A., Cuozzo, C., Sacchetti, S., Sasso, A., Chiariotti, L., Malorni, A., Abbondanza, C., and Avvedimento, E. V. (2008) DNA oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression. *Science* **319**, 202-206
129. Ogino, H., Nozaki, T., Gunji, A., Maeda, M., Suzuki, H., Ohta, T., Murakami, Y., Nakagama, H., Sugimura, T., and Masutani, M. (2007) Loss of Parp-1 affects gene expression profile in a genome-wide manner in ES cells and liver cells. *BMC Genomics* **8**, 41
130. Kraus, W. L. (2008) Transcriptional control by PARP-1: chromatin modulation, enhancer-binding, coregulation, and insulation. *Curr Opin Cell Biol* **20**, 294-302
131. Kim, M. Y., Mauro, S., Gevry, N., Lis, J. T., and Kraus, W. L. (2004) NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. *Cell* **119**, 803-814
132. Wacker, D. A., Ruhl, D. D., Balagamwala, E. H., Hope, K. M., Zhang, T., and Kraus, W. L. (2007) The DNA binding and catalytic domains of poly(ADP-ribose) polymerase 1 cooperate in the regulation of chromatin structure and transcription. *Mol Cell Biol* **27**, 7475-7485
133. Rouleau, M., Aubin, R. A., and Poirier, G. G. (2004) Poly(ADP-ribosyl)ated chromatin domains: access granted. *J Cell Sci* **117**, 815-825
134. Butler, A. J., and Ordahl, C. P. (1999) Poly(ADP-ribose) polymerase binds with transcription enhancer factor 1 to MCAT1 elements to regulate muscle-specific transcription. *Mol Cell Biol* **19**, 296-306
135. Nirodi, C., NagDas, S., Gygi, S. P., Olson, G., Aebersold, R., and Richmond, A. (2001) A role for poly(ADP-ribose) polymerase in the transcriptional regulation of the melanoma growth stimulatory activity (CXCL1) gene expression. *J Biol Chem* **276**, 9366-9374

136. Hassa, P. O., Buerki, C., Lombardi, C., Imhof, R., and Hottiger, M. O. (2003) Transcriptional coactivation of nuclear factor-kappaB-dependent gene expression by p300 is regulated by poly(ADP)-ribose polymerase-1. *J Biol Chem* **278**, 45145-45153
137. Hassa, P. O., Haenni, S. S., Buerki, C., Meier, N. I., Lane, W. S., Owen, H., Gersbach, M., Imhof, R., and Hottiger, M. O. (2005) Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription. *J Biol Chem* **280**, 40450-40464
138. Ju, B. G., Solum, D., Song, E. J., Lee, K. J., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (2004) Activating the PARP-1 sensor component of the groucho/ TLE1 corepressor complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway. *Cell* **119**, 815-829
139. Pavri, R., Lewis, B., Kim, T. K., Dilworth, F. J., Erdjument-Bromage, H., Tempst, P., de Murcia, G., Evans, R., Chambon, P., and Reinberg, D. (2005) PARP-1 determines specificity in a retinoid signaling pathway via direct modulation of mediator. *Mol Cell* **18**, 83-96
140. Klenova, E., and Ohlsson, R. (2005) Poly(ADP-ribosyl)ation and epigenetics. Is CTCF PARt of the plot? *Cell Cycle* **4**, 96-101
141. Hassa, P. O., and Hottiger, M. O. (2005) An epigenetic code for DNA damage repair pathways? *Biochem Cell Biol* **83**, 270-285
142. Juarez-Salinas, H., Sims, J. L., and Jacobson, M. K. (1979) Poly(ADP-ribose) levels in carcinogen-treated cells. *Nature* **282**, 740-741
143. Pleschke, J. M., Kleczkowska, H. E., Strohm, M., and Althaus, F. R. (2000) Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. *J Biol Chem* **275**, 40974-40980
144. Poirier, G. G., de Murcia, G., Jongstra-Bilen, J., Niedergang, C., and Mandel, P. (1982) Poly(ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure. *Proc Natl Acad Sci U S A* **79**, 3423-3427
145. Realini, C. A., and Althaus, F. R. (1992) Histone shuttling by poly(ADP-ribosylation). *J Biol Chem* **267**, 18858-18865
146. Woodhouse, B. C., and Dianov, G. L. (2008) Poly ADP-ribose polymerase-1: an international molecule of mystery. *DNA Repair (Amst)* **7**, 1077-1086
147. Timinszky, G., Till, S., Hassa, P. O., Hothorn, M., Kustatscher, G., Nijmeijer, B., Colombelli, J., Altmeyer, M., Stelzer, E. H., Scheffzek, K., Hottiger, M. O., and Ladurner, A. G. (2009) A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation. *Nat Struct Mol Biol* **16**, 923-929
148. Tsutsumi, M., Masutani, M., Nozaki, T., Kusuoka, O., Tsujiuchi, T., Nakagama, H., Suzuki, H., Konishi, Y., and Sugimura, T. (2001) Increased susceptibility of poly(ADP-ribose) polymerase-1 knockout mice to nitrosamine carcinogenicity. *Carcinogenesis* **22**, 1-3
149. Tong, W. M., Ohgaki, H., Huang, H., Granier, C., Kleihues, P., and Wang, Z. Q. (2003) Null mutation of DNA strand break-binding molecule poly(ADP-ribose) polymerase causes medulloblastomas in p53(-/-) mice. *Am J Pathol* **162**, 343-352
150. Conde, C., Mark, M., Oliver, F. J., Huber, A., de Murcia, G., and Menissier-de Murcia, J. (2001) Loss of poly(ADP-ribose) polymerase-1 causes increased tumour latency in p53-deficient mice. *EMBO J* **20**, 3535-3543
151. Martin-Oliva, D., Aguilar-Quesada, R., O'Valle, F., Munoz-Gamez, J. A., Martinez-Romero, R., Garcia Del Moral, R., Ruiz de Almodovar, J. M., Villuendas, R., Piris, M. A., and Oliver, F. J. (2006) Inhibition of poly(ADP-ribose) polymerase modulates tumor-related gene expression, including hypoxia-inducible factor-1 activation, during skin carcinogenesis. *Cancer Res* **66**, 5744-5756
152. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell* **100**, 57-70
153. Bolderson, E., Richard, D. J., Zhou, B. B., and Khanna, K. K. (2009) Recent advances in cancer therapy targeting proteins involved in DNA double-strand break repair. *Clin Cancer Res* **15**, 6314-6320
154. Ashworth, A. (2008) A synthetic lethal therapeutic approach: poly(ADP) ribose polymerase inhibitors for the treatment of cancers deficient in DNA double-strand break repair. *J Clin Oncol* **26**, 3785-3790
155. Fong, P. C., Boss, D. S., Yap, T. A., Tutt, A., Wu, P., Mergui-Roelvink, M., Mortimer, P., Swaisland, H., Lau, A., O'Connor, M. J., Ashworth, A., Carmichael, J., Kaye, S. B., Schellens, J. H., and de Bono, J. S. (2009) Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* **361**, 123-134

156. Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N. J., and Helleday, T. (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**, 913-917
157. Farmer, H., McCabe, N., Lord, C. J., Tutt, A. N., Johnson, D. A., Richardson, T. B., Santarosa, M., Dillon, K. J., Hickson, I., Knights, C., Martin, N. M., Jackson, S. P., Smith, G. C., and Ashworth, A. (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **434**, 917-921
158. Mendes-Pereirea, A. M. (2009) Synthetic lethal targeting of PTEN mutant cells with PARP inhibitors. *EMBO Molecular Medicine*, 315-322
159. Martin, S., Wilkinson, K. A., Nishimune, A., and Henley, J. M. (2007) Emerging extranuclear roles of protein SUMOylation in neuronal function and dysfunction. *Nat Rev Neurosci* **8**, 948-959
160. Knipscheer, P., van Dijk, W. J., Olsen, J. V., Mann, M., and Sixma, T. K. (2007) Noncovalent interaction between Ubc9 and SUMO promotes SUMO chain formation. *EMBO J* **26**, 2797-2807
161. Hänni, S. (2008) Regulation of Poly-ADP-ribose Polymerase-1 and -2 by Lysine Acetylation. p. 122, University of Zürich, Zürich
162. Haenni, S. S., Hassa, P. O., Altmeyer, M., Fey, M., Imhof, R., and Hottiger, M. O. (2008) Identification of lysines 36 and 37 of PARP-2 as targets for acetylation and auto-ADP-ribosylation. *Int J Biochem Cell Biol* **40**, 2274-2283
163. Messner, S., Schuermann, D., Altmeyer, M., Kassner, I., Schmidt, D., Schar, P., Muller, S., and Hottiger, M. O. (2009) Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function. *FASEB J* **23**, 3978-3989
164. Elser, M., Borsig, L., Hassa, P. O., Erener, S., Messner, S., Valovka, T., Keller, S., Gassmann, M., and Hottiger, M. O. (2008) Poly(ADP-ribose) polymerase 1 promotes tumor cell survival by coactivating hypoxia-inducible factor-1-dependent gene expression. *Mol Cancer Res* **6**, 282-290
165. Altmeyer, M., Messner, S., Hassa, P. O., Fey, M., and Hottiger, M. O. (2009) Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites. *Nucleic Acids Res* **37**, 3723-3738
166. Blomster, H. A., Hietakangas, V., Wu, J., Kouvonen, P., Hautaniemi, S., and Sistonen, L. (2009) Novel proteomics strategy brings insight into the prevalence of SUMO-2 target sites. *Mol Cell Proteomics*
167. Martin, N., Schwamborn, K., Schreiber, V., Werner, A., Guillier, C., Zhang, X. D., Bischof, O., Seeler, J. S., and Dejean, A. (2009) PARP-1 transcriptional activity is regulated by sumoylation upon heat shock. *EMBO J* **28**, 3534-3548
168. Stilmann, M., Hinz, M., Arslan, S. C., Zimmer, A., Schreiber, V., and Scheidereit, C. (2009) A nuclear poly(ADP-ribose)-dependent signalosome confers DNA damage-induced IkappaB kinase activation. *Mol Cell* **36**, 365-378
169. Stehmeier, P., and Muller, S. (2009) Phospho-regulated SUMO interaction modules connect the SUMO system to CK2 signaling. *Mol Cell* **33**, 400-409
170. Hassa, P. O., Covic, M., Hasan, S., Imhof, R., and Hottiger, M. O. (2001) The enzymatic and DNA binding activity of PARP-1 are not required for NF-kappa B coactivator function. *J Biol Chem* **276**, 45588-45597
171. Kolthur-Seetharam, U., Dantzer, F., McBurney, M. W., de Murcia, G., and Sassone-Corsi, P. (2006) Control of AIF-mediated cell death by the functional interplay of SIRT1 and PARP-1 in response to DNA damage. *Cell Cycle* **5**, 873-877
172. Rajamohan, S. B., Pillai, V. B., Gupta, M., Sundaresan, N. R., Birukov, K. G., Samant, S., Hottiger, M. O., and Gupta, M. P. (2009) SIRT1 promotes cell survival under stress by deacetylation-dependent deactivation of poly(ADP-ribose) polymerase 1. *Mol Cell Biol* **29**, 4116-4129
173. Zhang, J. (2003) Are poly(ADP-ribosyl)ation by PARP-1 and deacetylation by Sir2 linked? *Bioessays* **25**, 808-814
174. van Breukelen, B., Georgiou, A., Drugan, M. M., Taouatas, N., Mohammed, S., and Heck, A. J. LysNDeNovo: an algorithm enabling de novo sequencing of Lys-N generated peptides fragmented by Electron Transfer Dissociation. *Proteomics*
175. Ogata, N., Ueda, K., Kagamiyama, H., and Hayaishi, O. (1980) ADP-ribosylation of histone H1. Identification of glutamic acid residues 2, 14, and the COOH-terminal lysine residue as modification sites. *J Biol Chem* **255**, 7616-7620

176. Ogata, N., Ueda, K., and Hayaishi, O. (1980) ADP-ribosylation of histone H2B. Identification of glutamic acid residue 2 as the modification site. *J Biol Chem* **255**, 7610-7615
177. Brochu, G., Duchaine, C., Thibeault, L., Lagueux, J., Shah, G. M., and Poirier, G. G. (1994) Mode of action of poly(ADP-ribose) glycohydrolase. *Biochim Biophys Acta* **1219**, 342-350
178. Ahel, I., Ahel, D., Matsusaka, T., Clark, A. J., Pines, J., Boulton, S. J., and West, S. C. (2008) Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins. *Nature* **451**, 81-85
179. Till, S., and Ladurner, A. G. (2009) Sensing NAD metabolites through macro domains. *Front Biosci* **14**, 3246-3258
180. Ahel, D., Horejsi, Z., Wiechens, N., Polo, S. E., Garcia-Wilson, E., Ahel, I., Flynn, H., Skehel, M., West, S. C., Jackson, S. P., Owen-Hughes, T., and Boulton, S. J. (2009) Poly(ADP-ribose)-dependent regulation of DNA repair by the chromatin remodeling enzyme ALC1. *Science* **325**, 1240-1243
181. Gottschalk, A. J., Timinszky, G., Kong, S. E., Jin, J., Cai, Y., Swanson, S. K., Washburn, M. P., Florens, L., Ladurner, A. G., Conaway, J. W., and Conaway, R. C. (2009) Poly(ADP-ribosyl)ation directs recruitment and activation of an ATP-dependent chromatin remodeler. *Proc Natl Acad Sci U S A* **106**, 13770-13774
182. Vaquero, A., Scher, M. B., Lee, D. H., Sutton, A., Cheng, H. L., Alt, F. W., Serrano, L., Sternglanz, R., and Reinberg, D. (2006) SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes Dev* **20**, 1256-1261
183. Boulikas, T. (1990) Poly(ADP-ribosylated) histones in chromatin replication. *J Biol Chem* **265**, 14638-14647
184. Golderer, G., and Grobner, P. (1991) ADP-ribosylation of core histones and their acetylated subspecies. *Biochem J* **277 (Pt 3)**, 607-610
185. Zhou, J., Fan, J. Y., Rangasamy, D., and Tremethick, D. J. (2007) The nucleosome surface regulates chromatin compaction and couples it with transcriptional repression. *Nat Struct Mol Biol* **14**, 1070-1076
186. Clapier, C. R., Langst, G., Corona, D. F., Becker, P. B., and Nightingale, K. P. (2001) Critical role for the histone H4 N terminus in nucleosome remodeling by ISWI. *Mol Cell Biol* **21**, 875-883

Curriculum vitae

Personal Data:

Name:	**Simon Messner**
Date of birth:	17.11.1979
Citizenship:	Austria
Home town:	Breitenbach am Inn / Tirol

Education

Dec 2006 – Mar 2010	University of Zurich, Life Science Zürich Graduate School PhD-program in Molecular Life Sciences
Oct 2004 – Nov 2006	University of Innsbruck, Austria Master studies in Molecular biology and Microbiology
Oct 2001 – Oct 2004	University of Innsbruck, Austria Bachelor studies in biology
Sep 1994 – Jun 1999	Secondary school with focus on alpine agriculture (A-levels), Elixhausen, Austria

---

Professional Experience

Dec 2006 – Feb 2010	University of Zurich, Institute of Veterinary Biochemistry and Molecular Biology, Switzerland Research Associate in Biochemistry and Molecular Biology
May 2000 – Sept 2006	Agrarmarkt Austria, Innsbruck, Austria Controller of agricultural funds, part time from 2002-2006
Summer 2003 – 2004	Sandoz GmbH, Kundl, Austria Laboratory training in biotechnical production

---

Acknowledgements

I would like to thank my supervisor, Prof. Michael Hottiger, for support and motivation during the thesis project. I also thank the members of my thesis committee, Prof. Roland Wenger, Prof. Primo Schär and Dr. Vikram Panse, for agreeing to review my work and test my knowledge. I thank Prof. Ludger Hengst for agreeing to provide an external expertise of the thesis. I am also grateful to Dr. Stefan Müller, who was a great help with the SUMO-project. In addition, I want to thank all collaborators, especially Bernd, Peter and Doro from the FGCZ, who made mass spectrometry exciting for me. Furthermore, Prof. Amedeo Caflisch allowed me fascinating insights into the field of molecular dynamics, which I really appreciated very much.

I thank the Swiss National Foundation for financing my PhD studies.

A big thank you also to the IVBMB and its members for creating a great environment for work and also for great institute- and group-events. Many thanks to all labmates, especially Suheda, Matthias, Steffi, Enni, Ingrid and Karoline who helped me a lot with all kind of problems and helped me to enjoy the time at the institute. Many thanks also to the other members and former members of the institute, especially to Moni, Elena, Taras, Sandra, Michael, Lucy, Flurina and the new group members Florian and Andrew.
A very special thank you also to Andrea-Monika for continuously making me aware of the most important things in life.

Finally I would like to thank my family, especially my parents for unconditional support and encouragement.